AN ABSTRACT OF THE THESIS OF

Henry William Ortega for the degree of Master of Science in Microbiology presented on August 25, 1993.

Title: Mechanisms of Accessory Cell Function in Rainbow Trout (*Oncorhynchus mykiss*).

Redacted for Privacy

Abstract approved: _____.

Stephen L. Kaattari

The ability of different subpopulations of rainbow trout (*Oncorhynchus mykiss*) peripheral blood leukocytes to respond to the T-independent antigen trinitrophenylated-lipopolysaccharide (TNP-LPS) was assessed by using an *in vitro* leukocyte culture system, and cell partitioning techniques coupled with recent advances in limiting dilution analysis (LDA). It was demonstrated that macrophages are required for the provision of requsite accessory function during the development of an antibody response to this antigen. The need for this accessory function was demonstrated by the fact that macrophages (adherent leukocytes) were able to restore the capacity for antibody production to isolated lymphocytes (non-adherent leukocytes). Furthermore, supernatants from antigen- or lipopolysaccharide (LPS)-stimulated macrophages were sufficient to restore this lymphocyte function. Finally LDA confirmed this requisite role of the macrophage-derived factor, revealed that the target of this factor(s) is the B cell precursor, and suggests that there may be differential sensitivity of B cell precursors to this factor.

Biological activity of this trout monokine, as measured by the provision of accessory function in lymphocyte plaque forming cell (PFC) responses, is produced as early as 24 hours and at optimal levels by day 4 post LPS stimulation of leukocyte cultures.

Stimulation with high (200μg/ml) and low (0.2μg/ml) doses of LPS generates supernatants with different activities, as observed by polyclonal activation of lymphocytes. Stimulation of leukocytes with a low dose of LPS appears to induce the production of a factor which does not polyclonally activate lymphocytes, whereas a high dose of LPS induces a factor which polyclonally activates lymphocyte cultures. This suggests that there are at least two separate factors produced by leukocytes. Low dose LPS induces an IL-1-like accessory factor, whereas high dose LPS induces a polyclonal activating factor. These different activities were also demonstrated by the adsorption of supernatants onto paraformaldehyde-fixed lymphocytes. High dose LPS supernatant activity was reduced following adsorption with non-activated or antigen-activated lymphocytes, whereas low dose LPS supernatant accessory activity was reduced only when adsorbed with antigen-activated lymphocytes. This also suggests that lymphocyte surface receptors for the polyclonal activating factor are present on both antigen- and non-activated lymphocytes, while only activated lymphocytes express a surface receptor for the IL-1-like factor. Finally, the addition of these factors to lymphocytes at later stages of the PFC response decreases their efficiency in augmenting lymphocyte PFC responses. Thus the identification of two new salmonid cytokines is reported here. The similarities of the trout accessory factor and mammalian IL-1 have been discussed, and it is proposed that this is the first demonstration of a salmonid monokine analogous to mammalian IL-1. Based on the factor source, function, and apparent molecular weight it is postulated that this trout accessory factor is analogous to mammalian interleukin-1.

Mechanisms of Accessory Cell Function in Rainbow Trout (*Oncorhynchus mykiss*)

by

Henry William Ortega

A THESIS
Submitted to
Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed August 25, 1993
Commencement June 1994

APPROVED:

Redacted for Privacy

Professor of Microbiology in charge of major

Redacted for Privacy

Head of the Department of Microbiology

Redacted for Privacy

Dean of the Graduate School

Date thesis presented August 25, 1993 .

Typed by Henry William Ortega .

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and thanks to my major professor, Dr. Stephen Kaattari, for both the opportunity to work in his lab and the guidance in completing this thesis. Additionally I would like to thank Dr. Joann Leong and Dr. John Rohovec for useful and entertaining discussions throughout my graduate education at OSU.

My fellow lab members have been a source of inspiration, entertainment, and knowledge including Dr. Mark Adkison, Dr. Greg Wiens, Dr. Jenefer DeKoning-Loo, Jon Piganelli, John Hansen, Patty Wood, Janell Bishop, and Leslie Gilkey. I would also like to thank Dr. Reg McParland and the Central Services Lab for help with FPLC.

Specifically I would like to thank my mother, father, grandfather, brothers, and the rest of my family for unshakeable confidence, support, financial assistance, and love throughout my life. I would also like to thank Ms. Kelly Gonzales for her patience, support, and love while completing this thesis.

This research was funded by support from Oregon Sea Grant.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Mechanisms of Accessory Cell Function in Rainbow Trout (*Oncorhynchus mykiss*)

CHAPTER 1

Literature review

B Cells and the B Cell Response

Mammalian B cell ontogeny. It has been well documented that the sites of primary production of mammalian lymphoid, myeloid and erythroid precursors, include the bone marrow, liver and spleen in fetuses, and the bone marrow in adults (Owen et al., 1977; Levitt and Cooper, 1980; Whitlock et al., 1985). Differentiation of the lymphoid population then begins in the bone marrow which produces pre-B and pre-T cells (LePault, 1983). Pre-T cells subsequently migrate to the thymus, which is the primary site of T cell development and maturation (Owen, 1972). The population of immature lymphocytes containing cytoplastmic immunoglobulin M (cIgM), but lacking detectable surface Ig, termed pre-B cells, have been found in livers of 11-12 day mice, 7 week human and 21 day rabbit fetuses several days before surface IgM-positive B cells appear (Raff et al., 1976; Gathings et al., 1977; Andrew and Owen, 1978; Hayward et al., 1978). These and other studies have provided evidence that these cells are the precursors of B lymphocytes (Whitlock et al., 1985; Freitas et al., 1982).

In a normal, unmanipulated adult mouse, the bone marrow produces very large numbers of B lymphocytes. Although still debatable, it is believed that the bulk of B cell generation in the

bone marrow is solely guided by endogenous factors, and is independent of environmental influences. The rate of bone marrow B cell production is sufficiently high that within 2-4 days enough new B lymphocytes are produced to replenish the whole periphery of the mouse (Freitas et al., 1989; Osmond, 1986). These newly formed B cells are immunocompetent, express surface Ig, and respond to activation stimuli. These B cells subsequently migrate to the peripheral blood, lymph nodes and spleen, where upon further maturation, become surface IgM^+ / IgD^+, sensitive to antigen and mitogen activation, and are found in the adult spleen as the primary resting B-cell population.

Mammalian B cell response. The entire sequence of events leading to a B cell response to antigen is generally categorized into three stages: activation, proliferation, and differentiation. These stages of B cell development have been viewed as sequential events, initiated and regulated by distinct and specific signals (Dutton and Swain, 1987; Jelinek and Lipsky, 1987). Classically, B cell responses induced by various stimuli have been differentiated as either T cell independent (TI) or T cell dependent (TD) based on their apparent requirement for T cell factors to support the differentiation into antibody-forming cells. Today, this sequence of events is no longer so clearly defined. Recent studies suggest a variety of T cell derived lymphokines may be involved in each step of the B cell response, including preparing small resting B cells to respond before interaction of surface Ig receptors (Oliver et al., 1985; Rabin et al., 1985). B cell responses may then vary only in the degree of T cell help required. Thus, each stage of the B cell response will be discussed in terms of the effects of the classically defined TI and TD antigens, rather than the categorical TI versus TD response.

Salmonid B cell ontogeny. In the teleost, the sites of lymphohematopoeitic activity include the kidney, thymus and spleen. In addition to these organs, lymphocytes can be found in the peripheral blood, gut-associated lymphoid tissue (GALT), and in the

lymphatic vessels that are differentiated from the vascular system (Zapata and Cooper, 1990; Pontius and Ambrosius, 1972).

During ontogenetical development, the thymus has been shown to precede the spleen and kidney in the appearance of lymphocytes, though hematopoeitic stem cells are already identifiable in the kidney well before the onset of lymphoid differentiation in the thymus (Ellis, 1977; Grace and Manning, 1980; Zapata, 1981). The thymus exists as a pair of lobes, one located on each side of the gill cavity (Chilmonczyk, 1983), and is believed to consist of thymic lymphocytes in varying degrees of maturation, in addition to some erythrocytes and macrophages (Yasutake and Wales, 1983).

Though differentiated lymphocytes may first appear in the thymus, it is currently believed that the anterior kidney is the primary hematopoietic organ in the teleost, and has been compared to mammalian bone marrow in function (Zapata, 1979; Irwin and Kaattari, 1986; Zapata and Cooper, 1990). Every line of hematopoietic differentiation has been observed in the kidney including the hypothetically pluripotent stem cells, as well as immature and mature red and white blood cells (Smith et al., 1970; Zapata, 1979; Al-Adhami and Kunz, 1976). The kidney can be divided into two distinct segments, an anterior and a posterior segment (Ellis and de Sousa, 1974; Zapata, 1979). The primary site of hematopoiesis is found in the anterior quarter of the kidney which contains lymphatic vessels and opens into the coelomic cavity.

Alternately, the spleen is suggested not to be essential for immunological maturation and in most fish serves as an accessory hematopoietic organ. This has been evidenced by the studies demonstrating that kidney and thymus lymphocytes display surface immunoglobulin and respond in a mixed lymphocyte reaction even when the spleen is present only in an immature form (Ellis, 1977; Tatner and Manning, 1983; Yasutake and Wales, 1983). Additionally, the spleen when compared to the anterior kidney has been shown to harbor B cells comprised of a different repertoire than those found in the anterior kidney (Kaattari and Irwin, 1985). Splenic lymphoid

tissue is also not highly developed in teleosts. It differs from the mammalian spleen in that the red and white pulps are diffuse and connective tissue is not prominent (Robertson and Wexler, 1960; Anderson, 1974).

Salmonid B cell response. Phylogenetic studies generally agree that the earliest manifestations of immunoglobulins and antibody function is seen in fish (McKinney et al., 1977; Sima and Vetvicka, 1990). Initial assessment of a teleost humoral immune response *in vitro* can probably be attributed to Smith et al., 1967. Using a modification of the hemolytic plaque assay, cells producing hemolytic antibody were found in two organs, the spleen and the pronephros in the bluegill, *Lapomis macrochirus.* Using similar techniques, immunocompetent cells were later found in the spleen and anterior kidney of the rainbow trout, *Salmo gairdneri* (Chiller et al., 1969a; Chiller et al., 1969b; Pontius and Ambrosius, 1971). These studies demonstrated both antigen binding and antibody producing cells could be found in these organs. The ability to produce antibodies led to the conclusion that these fish possessed cells equal in at least one function to the B lymphocytes of mammals. But early studies conducted by many investigators could not distinguish if B lymphocytes in fish were specialized and therefore separate from T cells (or cells with T-like functions) or if B lymphocytes were multifunctional and served in both capacities as B and T cell combined.

What confused some early studies was the fact that the methods employed to identify B and T cells used polyclonal antisera raised against fish immunoglobulin. These antisera, being polyclonal, invariably reacted with all fish lymphocytes and thus was ultimately useless (Clem et al., 1991). In contrast, rosette formation and nylon wool filtration were used with some success to separate two distinct types of mitogen-reactive, antigen-binding cells (Cuchens and Clem, 1977; Ruben et al., 1977).

Accessory Cells and the Immune Response

Immune responses in ectothermic vertebrates are attributable to cell-cell interactions similar to those seen in higher vertebrates (reviewed by Vallejo et al., 1992). In both channel catfish and rainbow trout, unequivocal evidence exists to document the hypothesis that counterparts of mammalian T and B cells are distinct lymphocyte populations defined both in terms of phenotype and function (reviewed by Kaattari, 1992; Chilmonczyk, 1992). In mammals, the induction of antigen-specific immune responses requires yet another set of cells, loosely referred to as accessory cells, which include blood monocytes, tissue and exudate macrophages (Unanue, 1984; Papadimitriou and Ashman, 1989), dendritic cells (Melief, 1989), and epidermal Langerhans cells (Streilein and Grammer, 1989). These cells provide the machinery for antigen processing and contain major histocompatability (MHC) molecules required for the presentation of processed antigen to specific lymphocytes (Unanue, 1984; Chesnut and Grey, 1985), thus they are also referred to as antigen-presenting cells (APC). This particular accessory function is also accomplished by B cells, and to a certain extent T cells (van Rooijen, 1990; Harding, 1991). Second, they also provide growth factors (such as interleukin 1 [IL-1]) which are necessary costimulatory signals for T cell activation (Weaver and Unanue, 1990). In this regard, rainbow trout and catfish monocytes (or macrophages) as well as several other lower vertebrate species have been found to secrete a soluble factor akin to IL-1 (Ortega and Kaattari, 1993a; Vallejo et al., 1991). In fact, the requirement for trout monocytes or macrophages for the induction of either lymphocyte mitogenesis or anti-hapten plaque-forming cell (PFC) responses *in vitro* can be substituted by the addition of exogenous trout IL-1, similar to the situation observed in both catfish and mammalian systems. Reports by Vallejo and coworkers (Vallejo et al., 1990; Vallejo et al., 1991) suggest that either

accessory cells other than monocytes or macrophages, i.e. B cells, are involved in processing and presentation; alternatively, processing and presentation of TD antigens are not required in the generation of catfish immune responses. The results of studies addressing this issue are discussed below.

Antigen trapping: in vivo studies. The hypothesis that antigen nonspecific cells participate in the induction of ectothermic vertebrate specific immune responses originally stemmed from studies on antigen retention in different tissues and organs. Early work by Kroese and colleagues (Kroese, 1983; Kroese et al., 1985) and White et al. (1975) invariably demonstrated that a wide variety of foreign substances (antigens such as soluble proteins, bacterins, and preformed immune complexes) were "trapped" in the spleen and other peripheral lymphoid organs of various vertebrate species. Histological and ultrastructural analysis showed that such trapped substances were consistently found associated with phagocytes (macrophages) within lymphoid organs (Ellis et al., 1976; Ellis, 1980). Similar observations were reported for different fish species (Secombes et al., 1982; Lamers and de Haas, 1985; Ziegenfuss and Wolke, 1991). It appears that phagocytic cells, particularly those in the spleen and pronephros, form discrete aggregates that increase in both size and number subsequent to administration of antigen (Zapata et al., 1987). It has been repeatedly demonstrated by researchers that regardless of tissue or organ location, such phagocytes are situated in the vicinity of populations of lymphocytes (Secombes et al., 1982; Lamers and de Haas, 1985), leading to the suggestion that these phagocytes are reservoirs of antigen and are involved in antigen presentation for the induction of specific antibody responses *in vivo* (Miller et al., 1987; Rombout et al., 1989).

While such a hypothesis is plausible, localization of antigen within phagocytes of lymphoid or hematopoietic organs in itself does not prove that such organs or cells are antigen reservoirs. For example, Szakal et al. (1983) have reported that murine

macrophages in the subcapsular and medullary sinuses of lymphoid tissues actually transport ingested antigen from the afferent to the efferent side of the organ for clearance. Moreover, early studies using both mammals (Nilsson and Berg, 1977) and fish (Smedsrud et al., 1984) have shown that considerably large proportions of antigens are localized in the liver and the excretory tissues of the kidneys suggesting active clearance or elimination of the foreign materials. Furthermore, Blazer et al. (1987) and Ziegenfuss and Wolke (1991) have reported that accumulations of particulate materials in piscine livers were found among macrophage aggregates similar to those observed in the spleen and kidneys. Although such antigen-trapping may result in the production of detectable quantities of specific serum or mucosal antibodies (Rombout et al., 1989), the exact role of tissue macrophages in antibody production cannot be determined solely by the use of such descriptive studies.

The existence of antigen accumulation *in vivo* has been demonstrated in the mouse by Szakal et al. (1988). Following antigen exposure, follicular dendritic cells in peripheral lymph nodes developed a "beaded" morphology. Immunocytochemical and ultrastructural analyses revealed that these "beads" contained antigen and persisted for long periods of time (up to 14 days). These beads were slowly released and dispersed into the surrounding microenvironment where germinal center B cells and resident macrophages were found to endocytose them (Szakal et al., 1988). The function of these putative antigen reservoirs was confirmed by Tew et al. (1988) with the demonstration that these bead-containing B cells and macrophages served as APC for the stimulation of IL-2 production by antigen-specific T cells *in vitro*. Further, it was demonstrated that murine macrophages can transfer processed antigen to B cells, which in turn present antigen to specific T cells.

Antigen uptake and catabolism: in vitro studies. Phagocytes, particularly macrophages, are actively involved in antigen clearance regardless of species; these cells are clearly among the first lines of

defense against infection (reviewed by Papadimitriou and Ashman, 1989). Without antigen uptake and processing immune responses would most likely be limited due to a decreased exposure to recognizable antigens. Definitive studies pertaining to the mechanisms of piscine phagocytosis, intracellular killing, and catabolism have been reported (reviewed by Secombes and Fletcher, 1992). Such studies were facilitated by the relative ease of isolation of fish macrophages and monocytes as reported by Miller and coworkers (Miller et al., 1985; Miller et al., 1986).

Direct correlation between antigen uptake and degradation by fish macrophages and subsequent induction of antigen-specific immune responses has yet only been unequivocally demonstrated in catfish by Vallejo and coworkers (Vallejo et al., 1990; Vallejo et al., 1991). These researchers have shown that uptake of exogenous protein antigens by freshly isolated catfish peripheral blood monocytes (fibronectin-adherent, nonspecific esterase positive cells) or long term cultures of such cells was subsequently followed by intracellular proteolysis. In addition, antigen uptake and degradation by monocytes were observed even at low temperature regimes previously reported to induce suppression of primary catfish T cell responses (Miller and Clem, 1984; Bly and Clem, 1991). Vallejo and coworkers (Vallejo et al., 1990) also report that following exposure to antigen, cells may be used as APC for the induction of antigen-specific immune responses *in vitro* by autologous immune lymphocytes. These findings indicate that catfish monocytes do not simply degrade antigens for the purpose of elimination, but that such metabolic activities result in the exposure of antigen determinants that are stimulatory to specific lymphocytes. Clearly, these observations support the idea that accessory cells are required for the generation of fish immune responses as previously reported by Miller, Clem and coworkers (Miller et al., 1985; Clem et al., 1985).

Antigen presentation. To further elucidate the role of accessory cells in fish immune responses, Clem and coworkers (Miller and

Clem, 1984; Vallejo et al., 1990) have reported observations which suggest that antigen presentation occurs and macrophages perform this function (Vallejo et al., 1991). Initial strategies utilized immune peripheral blood leukocytes (PBL) both as accessory cells and responders for the induction of secondary antigen-specific immune responses *in vitro* (Vallejo et al., 1990). Catfish PBL previously primed *in vivo* with antigen were pulsed with homologous antigen *in vitro*, washed, fixed with paraformaldehyde, and then co-cultured with freshly isolated autologous immune PBL. Antigen-pulsing of the putative APC allowed for antigen uptake and intracellular processing as observed in mammalian accessory cells (reviewed by Chain et al., 1988; Yewdell and Bennick, 1990). Therefore it was suggested by the authors that any antigen-specific immune responses elicited by autologous PBL responders were attributed to antigen which became APC-associated during antigen-pulsing. Furthermore, fixation of the putative APC ensured that such cells did not proliferate in culture and prevented further processing of antigen. The results of this study showed that immune PBL responders underwent antigen-specific proliferation and antibody production when cocultured with antigen-pulsed and fixed APC similar to control PBL cultures directly stimulated with soluble homologous antigen (Vallejo et al., 1990). These *in vitro* responses were abrogated by fixation of putative APC prior to antigen-pulsing.

The advent of cell separation protocols has permitted the identification of many immune cell functions in both mammalian and piscine systems, including the identification of the APC. Studies with catfish immune cells using similar antigen-pulsing strategies (with unfractionated PBL used as APC) showed that both monocytes and B cells are efficient APC (Vallejo et al., 1991). It should be noted that previous studies (Smith and Braun-Nesje, 1982; Sizemore et al., 1984; Graham and Secombes, 1990) have determined that the number of macrophages necessary to give optimal responses *in vitro* is in the order of 1-8% of the responding population, with larger numbers of macrophages resulting in no further significant effect. Antigen-pulsed and fixed T cells also stimulated the proliferation of

autologous PBL responders, although the magnitude of the responses was considerably lower (approximately 10% of the maximum response) than those elicited with either monocytes or B cells (Vallejo et al., 1991). The finding that catfish T cells have weak APC capacity was not particularly suprising. In mammals, T cells may express MHC molecules, the self-determinants responsible for antigen presentation and immune restriction. Such MHC-expressing T cells have been shown by Reske-Kunz et al. (1986) to be effective APC. Although the exact role of MHC (or MHC-like) molecules in antigen presentation in piscine systems has yet to be elucidated, the apparent restrictions of catfish immune responses discussed above suggest that piscine T cells may be viable APC, albeit less efficient than either monocytes or B cells.

Cell separation techniques have also been used to determine the accessory cell-derived requirements of a number of other piscine immune responses. In Atlantic salmon and catfish, such experiments have shown that T cell mitogen and mixed leukocyte reaction (MLR) responses are dependent upon the presence of macrophages (Smith and Braun-Nesje, 1982; Sizemore et al., 1984; Miller et al., 1986). Similarly, catfish APC responses to TD and TI antigens are accessory cell dependent (Miller et al., 1985), as is lymphokine (macrophage activating factor) production by surface Ig negative (sIg-) lymphocytes in rainbow trout (Graham and Secombes, 1985). Ellsaesser et al. (1988) have shown that a naturally occurring accessory cell depleted lymphocyte population appears to be present in the catfish thymus, where isolated thymocytes are unable to proliferate *in vitro* in response to Con A unless monocytes are added to the cultures. In an alternative approach, Sizemore et al. (1984) have shown that inhibition of macrophage function with 5mM L-leucine methylester markedly decreases the mitogenic response of unfractioned leukocytes to Con A. These responses can be restored by adding untreated macrophages from the same individual, confirming the selectivity of the effect. To date, only the mitogen responsiveness of sIg+

lymphocytes has been shown to be macrophage-independent (Sizemore et al., 1984).

Indications that accessory cell function may be genetically restricted have been presented by Vallejo et al. (1990) by using allogeneic leukocytes as antigen presenting cells. Although able to stimulate a proliferative (MLR) response, antigen-pulsed allogeneic cells cannot induce an antibody response from *in vivo* primed cells. It has been reported, however, that on the rare occasions when two way MLR negative pairs of fish have been found (indicative of small or no MHC differences), mixing allogeneic cells does allow antibody responses to occur with homologous cells (Clem et al., 1991).

Cellular pathways of antigen processing and presentation. Studies with mammalian models have revealed that antigen processing involves a complex series of normal cellular and biochemical events that result in the exposure of epitopes that are otherwise buried in the native conformation of an antigen (reviewed by Chain et al., 1988). Such events may involve simple unfolding, denaturation, and partial or complete proteolysis, and may occur either intracellularly or on the plasma membrane of accessory cells (Harding et al., 1988; Chain et al., 1988; Semple et al., 1989). Regardless of the mode and site of processing, antigens become structurally modified to a particular conformation capable of binding MHC molecules (Harding et al., 1990). This latter bimolecular complex is re-expressed on the surface of accessory cells and presented to specific T cells (Braciale et al., 1987). In mammals, there are two pathways of antigen processing that are closely tied to the two classes of MHC molecules and the two T cell subsets (Harding, 1991). There is the endogenous or cytosolic pathway and the exogenous or endocytic pathway. In the exogenous pathway, antigens are endocytosed by the APC from its environment. Antigens are then processed intracellularly in an acidic endocytic compartment and subsequently intersect with endosomes containing Class II MHC molecules *en route* to the plasma membrane (Jensen, 1990). Harding et al. (1990) have shown that the acidic environment of the endosome may facilitate the

interaction of MHC with the appropriate processed antigen or immunogenic peptides.

In contrast, the endogenous pathway is primarily tied to Class I MHC binding of antigens (Harding, 1991). It is commonly accepted that cytotoxic T cells (i.e. CD8+ cells) are able to recognize their targets by this pathway. In this case, APC synthesize the antigens, which are mostly tumor and viral proteins. It has been reported by Yewdell and Bennick (1989) that such antigens are then targeted to endosomes where they undergo processing subsequently leading to the interaction of immunogenic peptides with Class I MHC molecules.

Although there is clear dichotomy of processing pathways of antigens and their presentation by two classes of MHC molecules, they are by no means absolute. Several studies reveal that exogenous antigens may also be presented by Class I MHC molecules and conversely, endogenous antigens may also be presented by Class II MHC molecules (Nuchtern et al., 1990; Rock et al., 1990). The factors that determine which class of mammalian MHC molecules the "processed" antigen binds are not known, although there is recent evidence that the invariant chain of Class I might have such a role (Teyton et al., 1990).

The results of recent piscine studies on antigen processing and presentation in the channel catfish reported by Vallejo and coworkers exhibit hallmarks of the exogenous pathway (Vallejo et al., 1990; Vallejo et al., 1992). Clearly, catfish APC take up a variety of exogenous protein antigens which are then subjected to intracellular degradation. Moreover, the observation that antigen-pulsing of viable, but not pre-fixed, APC results in the formation of peptide fragments of both complex and simple TD antigens (Vallejo et al., 1991) certainly indicates that antigen processing is a necessary step in the immunological recognition of foreign antigens in fish. Finally, the importance of intracellular proteases is also demonstrated by the finding that catfish APC functions are also blocked in the presence of protease inhibitors during antigen-pulsing (Vallejo et al., 1991).

Production of cytokines with accessory activity. Another critical aspect of accessory cell function is the elaboration of cytokines which mediate immune cell interactions. Particularly important are those cytokines which mediate natural immunity, and those which regulate lymphocyte activation, growth, and differentiation. The cytokines involved in these processes are the interferons, interleukin-1 (IL-1), and interleukin-2 (IL-2). The role of these cytokines in both mammalian and piscine immune responses are introduced and discussed below.

Cytokines and the Immune Response

The immunological literature of the 1960s and 1970s is replete with descriptions of different *in vitro* biological activities of "factors" (cytokines) in culture supernatants of antigen- and mitogen-stimulated leukocytes. Such investigations resulted in a veritable alphabet soup of acronyms to describe the diverse effects of these leukocyte-derived factors on the *in vitro* activities of leukocytes (such as Macrophage Inhibition Factor [MIF], Macrophage Activating Factor [MAF], and Lymphocyte Activating Factor [LAF]) (Dumonde et al., 1969; Neta et al., 1990). It was not until recombinant DNA technology made it possible to produce large quantities of the different cytokines that some order in nomenclature was achieved and an understanding of cytokine function was forthcoming. Currently, at least 16 different human cytokines have been isolated, their primary structure determined, their genes cloned, and their biological effects examined (reviewed by Neta et al., 1990). It is now known that effective communication among leukocytes during the course of an immune or inflammatory response depends on these low molecular weight (Mw) polypeptide cytokines. It is now well accepted that cytokines are involved in regulating the size and duration of immune and inflammatory responses, are produced both transiently and locally, and can act in a paracrine, autocrine, and possibly an endocrine fashion. Previous studies (reviewed by Neta et al., 1990) have revealed considerable duplication of *in vitro* biological activity by biochemically and genetically distinct cytokine molecules. These studies have also pointed out that cytokines interact in a network in which one cytokine can induce or inhibit the production of another, as well as modulate the action of other cytokines on the same target cell.

Cytokines produced predominantly by cells of the macrophage/monocyte lineage are termed monokines and include the type 1 interferons (IFN) alpha and beta (Friedman and Vogel,

1983), interleukin-1 (IL-1) (diGiovine and Duff, 1990; Dinarello, 1990), IL-6 (Kishimoto et al., 1990), tumor necrosis factor (TNF) alpha (Beutler and Cerami, 1988), and the granulocyte and monocyte colony-stimulating factors (G-CSF, M-CSF, GM-CSF) (Kelso et al., 1990). Cytokines produced by lymphocytes are termed lymphokines and include transforming growth factor beta (TGFB) (Derynck et al., 1985), IFN gamma (Trinchieri and Perussia, 1985), IL-2 (Smith, 1984), IL-3 (Schrader, 1986), IL-4 (Paul and Ohara, 1987), IL-5 (Kinashi et al., 1986), IL-7 (Namen et al., 1988), and lymphotoxin (TNF beta) (Paul and Ruddle, 1988).

The vast majority of the past and current cytokine literature is devoted exclusively to cytokines of human or murine origin that are assayed in systems involving human or murine cells and tissues. Most of the remaining cytokine literature involves other mammalian species. There are, however, studies that address the issue of whether leukocytes of nonmammalian vertebrates also produce cytokines. This review will focus on those regulatory cytokines which have provisionally been identified in teleost species as interferons, IL-1, and IL-2, by virtue of their biochemical and functional similarities to those molecules in mammals. In this review only mammalian and piscine cytokines will be addressed.

Interferons in mammalian immune systems. Interferons were originally defined by their ability to inhibit virus replication (Wheelock, 1965; Trinchieri and Perussia, 1985). In mammals, three major classes of interferon have been described: IFN alpha, beta, and gamma (reviewed by Stewart, 1980; Farrar and Schreiber, 1993). IFN alpha and beta are sometimes grouped together as type I interferons. Molecular cloning of these interferons has revealed more than 25 subtypes of IFN alpha and 2 of IFN beta (Henco et al., 1985; DeGrado et al., 1991).

Both IFN alpha and beta are produced by a variety of cell types including macrophages, endothelial and epithelial cells, and lymphocytes (Pestka et al., 1987). However, IFN alpha and beta are produced predominantly by macrophages and fibroblasts,

respectively (Stewart, 1980). Taniguchi et al. (1980) have reported a 45% nucleotide sequence identity between cloned human IFN alpha and beta genes, suggesting they could have originated from a common ancestral gene several hundred million years ago. Divergence may have occurred after the appearance of bony fish, since no cross-hybridization occurs between human IFN alpha cDNA and fish DNA, although weak hybridization with human IFN beta does occur (Wilson et al., 1983).

Type I interferons have a variety of *in vitro* and *in vivo* effects in addition to their antiviral activities. *In vitro,* type I interferons inhibit cell proliferation, tumor growth, and fibroblast differentiation, as well as stimulate macrophages accessory activity and cytokine production (reviewed by Rhodes et al., 1986). *In vivo,* type I interferons can modulate antibody production, delayed hypersensitivity reactions, and induce inflammatory reactions (Farrar and Schreiber, 1993).

IFN gamma, the product of antigen or mitogen activated T cells and natural killer (NK) cells (Kasahara et al., 1983; Munakata et al., 1985), is unrelated to type I interferons; IFN gamma displays less than 10% amino acid sequence identity with IFN alpha or beta (Taniguchi et al., 1980; Gray and Goeddel, 1982; Gray and Goeddel, 1983). Nevertheless, like type I interferons IFN gamma modulates macrophage functions by activating macrophage oxidative metabolism and antimicrobial activity(Schreiber et al., 1983). IFN gamma can also upregulate expression of class I and II MHC antigens on a variety of cell types (reviewed by Inaba et al., 1986).

Interleukin-1 in mammalian immune systems. Originally named Lymphocyte Activating Factor (LAF), interleukin 1 (IL-1) is often also called by many other names which describe this factor's different reported activities (reviewed by Neta et al., 1990).

Two forms of IL-1 have been isolated: IL-1 alpha (Gubler et al., 1986) and IL-1 beta (Matsushima et al., 1986). Both of these polypeptides have been found in human, rabbit (Furutani et al., 1985), and mouse (Gray et al., 1986) and exhibit significant

interspecies homology at the amino acid level (62% homology between human and mouse IL-1 alpha: Gray et al., 1986). Mature IL-1 alpha and IL-1 beta are both polypeptides with molecular weights of about 17kD (Dinarello, 1985; March et al., 1985). The significant variation observed in the literature presumably is due to the heterogenity of glycosylation of both forms. Perhaps the most distinguishing characteristic of these two peptides is their isoelectric points (pI): the pI of IL-1 alpha is 5.0 whereas the pI of IL-1 beta is 7.0 (Ihrie and Wood, 1985; Matsushima et al., 1986). Although neither IL-1 alpha nor beta display strict species specificity, the intensity of biological responses appears to be species specific (Ellsaesser, 1989; Simon and Lee, 1986).

IL-1 alpha and beta represent the products of two distinct genes and are each initially synthesized as 30-32kD precursor peptides of 270 and 269 amino acids, respectively (Hazuda et al., 1991). Mature forms of IL-1 alpha and beta are represented by C-terminal cleavage products of 159 and 153 amino acids, respectively (March et al., 1985). Tocci and collegues (Tocci et al., 1985; Cameron et al. 1985) have demonstrated that both IL-1 precursors lack a hydrophobic signal peptide thought to be required for the extracellular transport of proteins manufactured within the cell. Recent evidence reported by Beuscher and collegues (Beuscher et al., 1988; Beuscher and Colten, 1988) suggests that phosphorylation of human and mouse IL-1 alpha precursors may play a role in processing and transport via a lysosomal pathway. Biochemical data reported by Zola et al. (1993) demonstrate the existence of a membrane bound form of IL-1 alpha. However, the mechanism of IL-1 cell secretion is still largely unknown.

An inhibitor of IL-1 has recently been identified (Arend, 1991; Conti et al., 1992a). This molecule, termed IL-1 receptor antagonist (IL-1ra), binds to the IL-1 receptor with similar affinities as IL-1, but has no IL-1-like activity even at high concentrations, thus accounting for its antagonistic properties (Conti et al., 1992c). The purified molecule has a molecular weight of approximately 18-22kD, compared with a predicited molecular weight of 18kD from

cloning data (Arend, 1991). It is believed that glycosylation patterns account for the differences in molecular weight. IL-1ra appears to be produced by the same cells which produce IL-1 alpha and beta (Re et al., 1993; Andersson et al, 1992). It has limited sequence similarity at the amino acid level to IL-1 alpha (19%) and beta (26%) (Conti et al., 1992b). IL-1ra is induced in monocytes by IgG, LPS, PMA, and zymosan A (Re et al., 1993). Unlike IL-1 alpha or beta, IL-1ra appears to be species-specific in its binding to the IL-1 receptor (Hannum et al., 1990).

IL-1 alpha and beta exert their effects by binding to two distinct receptor types (Spriggs et al., 1992). The type I IL-1 receptor is an 80kD protein (Chizzonite et al., 1989; Bomsztyk et al., 1989) and is a member of the Ig superfamily of receptors, possessing three extracellular Ig-like domains, a short transmembrane domain, and a relatively large cytoplasmic domain (Spriggs et al., 1992). Both human and murine versions of this receptor have been identified and exhibit similar characteristics in terms of affinity for IL-1 and receptor number (Bomsztyk et al., 1989). Sequence information obtained for the human IL-1 receptor type I reported by Spriggs et al. (1992) indicates a 69% amino acid homology between the human and murine forms of this receptor.

A second IL-1 binding protein, called IL-1 type II receptor has also been cloned (McMahan et al., 1991). This protein, also a member of the Ig superfamily, has an extracellular ligand-binding domain composed of three Ig-like domains, a single transmembrane domain, and a short cytoplasmic domain of 29 amino acids in contrast to a large 215 amino acid cytoplasmic domain of the type I IL-1 receptor (McMahan et al., 1991). Its predicted molecular weight from the cloned cDNA is approximately 44kD, compared with the molecular weight of the purified protein of 60-68kD. Glycosylation at several residues probably account for these differences (Mancilla et al., 1992). The type II receptor is expressed on a number of different tissues, including both T and B cells, monocytes, granulocytes, macrophages, and neutrophils (Spriggs et al., 1992). IL-1 type II receptor also binds to IL-1 alpha, beta, and

IL-1ra with different affinities as compared with the type I receptor (McMahan et al., 1991).

Both human and murine receptors appear to signal via activation of a phosphorylation cascade, although both lack intrinsic kinase activity (McMahan et al., 1991). Portions of the cytoplasmic domain of the receptor seem to share sequence similarities with known nucleotide binding proteins, suggesting that IL-1 dependent signals may involve an intrinsic receptor G protein-like activity (McMahan et al., 1991). IL-1 has been shown to increase phospholipase A2 activity and the release of linoleic acid from certain T cells (Bomalski et al., 1992). Weitzman and Savage (1992) have reported observations indicating IL-1 receptor complexes associated with the nucleus following incubation of target cells with IL-1. IL-1 receptor activation also activates both the AP-1 and NF-kB transcription factors (Iwasaki et al., 1992).

A number of cell types have been shown to produce IL-1, including monocyte and macrophage cell lines, natural killer cells (NK), B cell lines, B lymphoblasts, endothelial cells, melanoma cell lines, neutrophils, fibroblasts, epithelial cells, adult T cell leukemic cell lines, and certain murine T cell lines (reviewed by Dinarello, 1990). Some reported IL-1 activities include (reviewed by Radeke et al., 1991):

1. Activation of T cells in the presence of co-stimulation through the T cell receptor (Mizuochi et al., 1988).

2. Induction of IL-2 receptor expression and cytokine gene expression (Lowenthal et al., 1986; Conti et al., 1992b).

3. Co-stimulation of thymocyte proliferation (Herbelin et al., 1992).

4. Stimulation of pre-B cell differentiation (Oka and Ito, 1987).

5. Co-stimulation of B cell proliferation and Ig secretion (Bonnefoy et al., 1989).

6. Augmentation of IL-2 and IFN-induced activation of NK-mediated cytotoxicity (Marumo et al., 1992).

7. Modulation of reparative functions following tissue injury (Neta et al., 1991; Kessler et al., 1992).

8. Induction of acute phase protein synthesis by hepatocytes (Brevario et al., 1992).

It has also been shown that IL-1 stimulates the release of factors associated with the growth and differentiation of cells from myeloid and lymphoid lineages *in vitro* (Zoja et al., 1992; Streiter et al., 1992; Herbelin et al., 1992). Fibbe et al. (1988) have reported that IL-1 may induce the production of macrophage colony stimulating factor by human bone marrow stromal cells. Granulocyte-macrophage colony stimulating factor from both human fibroblasts and peripheral blood lymphocytes have also been shown to be induced by IL-1 (Fibbe et al., 1988; Allen et al., 1992). IL-1 also stimulates hematopoiesis by upregulating receptors for colony stimulating factors and inducing the proliferation of pluripotent progenitors in the bone marrow (Hestdal et al., 1992).

Interleukin-2 in mammalian immune systems. IL-2 was the first of a series of T-helper cell-derived lymphokines to be described and completely characterized at the protein (Robb et al., 1983; Welte et al., 1982) and nucleotide level (Holbrook et al., 1984). IL-2 (formerly called T Cell Growth Factor [TCGF]) plays a key role in antigen-specific clonal proliferation of T cells (reviewed by Smith, 1988; Sabath and Prystowsky, 1990). IL-2 also acts on activated B cells to induce growth and secretion of immunoglobulin (Harada et al., 1987; Nakanishi et al., 1987). IL-2 can also evoke superoxide production by activated macrophages (Holter et al., 1987), as well as increase killing activity of NK cells (Zarcone et al., 1987). Administration of purified IL-2 to humans in clinical trials has been shown induce a rise in plasma ACTH and cortisol, supporting the notion that IL-2 has hypothalamo-pituitary-adrenal axis activity *in vivo* (Lotze et al., 1985).

Human IL-2 isolated from cell culture supernatants has been shown to have a Mw of 19-22kD by gel filtration chromatography and 14-16kD as determined by SDS-PAGE (Robb and Greene, 1983). Cloning data obtained from IL-2 cDNA sequence suggest that IL-2 is originally translated as a 153 amino acid polypeptide (Taniguchi et

al., 1983; Nikaido et al., 1984). Cleavage of the 20 residue leader peptide sequence yields a 133 amino acid protein with a predicted Mw of 15.4kD (Taniguchi et al., 1983).

IL-2 exerts its effects through a specific, saturable receptor system found primarily on T cells, B cells, and NK cells. The IL-2 receptor is a multi-subunit system consisting of a 55kD alpha subunit (Tac antigen) and a 70kD beta subunit (Robb et al., 1984). Whereas the beta subunit is expressed on resting T cells, alpha subunit expression must be induced by mitogenic stimulation (Holter et al., 1987). A soluble form of IL-2 receptor has been identified by Kuo and collegues which is released by mitogen- or antigen-activated T cells (Kuo and Robb, 1986; Kuo et al., 1986). The N-terminal 192 amino acids of the membrane-bound alpha subunit is released by proteolytic cleavage from the T cell surface to form the 45kD soluble IL-2R (Neeper et al., 1987; Taniguchi et al., 1990).

IL-2 is produced by T cells in response to activation by mitogens, alloantigens or antigens presented in context with the appropriate MHC molecules (Tigges et al., 1989). Perhaps the most important effect of IL-2 is the autocrine or paracrine stimulation of T cells expressing IL-2 receptors reported by Taniguchi et al. (1990). Briefly, it is believed that T cell activation fits a competence and progression model. Competence is first conferred by stimulation of T cells via the T cell antigen receptor, which in the presence of other accessory signals (such as IL-1), promotes entry into the G1 phase of the cell cycle. Progression then follows and is dependent upon the expression of IL-2 and IL-2 receptor alpha subunit interaction of IL-2 with its receptor and the expression of other gene products required for entry into S phase (Tigges et al., 1989).

In addition to its well known effects on T cell growth, IL-2 has been shown to augment B cell growth and immunoglobulin production (Harada et al., 1987), augment interferon gamma production (Smith, 1988), induce IL-6 production by human monocytes (Kishimoto et al., 1987), and modulate the expression of the IL-2 receptor (Harada et al., 1987).

Interferons in piscine immune systems. Owing to the antiviral activities of the mammalian interferons and a number of known viral diseases of commercially important species of fish, there is substantial literature reflecting the search for interferon-like cytokines in teleosts (Okamoto et al., 1983). In some studies, antiviral activity was induced by the parenteral administration of pathogenic viruses and assayed by measuring the nonspecific protective effects of serum either *in vitro* on virus-infected fish cells (deKinkelin et al., 1982) and/or *in vivo* by passive transfer of the same serum from which antiviral antibodies had been removed (Desena and Rio, 1975). Characterization of partially purified serum components revealed that the molecules conferring antiviral activity had an apparent Mw from 24 to 94 kD with isoelectric focusing (IEF) points from 5.4 to 7.1 (Desena and Rio, 1975). These antiviral serum components appeared to be type I interferons by virtue of their temperature and acid stability.

Recently, better resolution of the factor(s) displaying IFN-like activity in fish has been provided by the sytematic studies of Graham and Secombes (1988). Culture of rainbow trout peripheral blood and head kidney leukocytes that had been pulsed for 3 hours with Con A resulted in increased spreading and adherence of macrophages (Secombes, 1987). Such experiments produced culture supernatants which contained apparent MAF activity in that they increased the respiratory burst (superoxide production) and bactericidal activity of either resident or elicited macrophages (Secombes, 1987). Greater MAF activity occurred when leukocytes were incubated with phorbol 12-myristate-13-acetate (PMA) together with Con A (Graham and Secombes, 1990a). In a recent study, Graham and Secombes (1990b) used cell adherence and panning cell purification procedures to demonstrate that supernatants from cultures of phorbol ester/lectin pulsed "pure" macrophages or extensively purified surface Ig^+ (B) cells lacked MAF activity. In contrast, significant MAF activity was present in supernatants produced by stimulated sIg^- (T) cells, providing that macrophages were also present in the cultures.

Interleukin-1 in piscine immune systems. In a series of *in vitro* investigations, Clem, Miller, and colleagues have demonstrated that in the channel catfish, monocytes are needed for antibody production and Con A and antigen-induced stimulation of peripheral blood leukocytes (PBLs) and thymocytes (Miller et al., 1986). Recently, Ellsaesser (1989) and Clem et al. (1991) have provided convincing evidence that 24 hour culture supernatants from LPS-treated catfish monocytes can replace monocytes in *in vitro* immunological assays. The cytokine(s) in these supernatants were also active in a mouse thymocyte costimulation assay. Treatment of the supernatants with polyclonal anti-human IL-1 alpha/beta antibodies partially inhibited their activity (Ellsaesser, 1989). Whether this incomplete inhibition means that other cytokines (such as IL-6) are also present in these supernatants is unknown.

Supernatants from a mouse macrophage cell line, P388D1, but not recombinant human or mouse IL-1, could also replace catfish monocytes in fish anti-TNP-LPS antibody responses and in Con A-induced proliferation assays of catfish monocyte-depleted thymocytes or PBLs (Ellsaesser, 1989). It is not known if murine supernatants can also replace monocytes in the *in vitro* responses of catfish cells to thymus-dependent (TD) antigens.

Isoelectric focusing (IEF) of channel catfish monocyte supernatants reported by Ellsaesser (1989) revealed two peaks of activity in a costimulation assay with catfish cells, with isoelectric focusing points (pIs) of 5 and 8. When the IEF fractions were assayed on mouse cells, three peaks of activity could be visualized (pIs of 3.5, 4.7, and 6.1). From these data Ellsaesser (1989) suggested that different molecules in the catfish monocyte supernatants were responsible for stimulating mouse IL-2 sensitive cells and catfish PBLs.

Western blot analysis of crude supernatants from fish and mouse cells, and of active fractions from gel filtration columns was performed using polyclonal antisera to human IL-1 alpha, beta, or mouse rIL-1 alpha (Ellsaesser, 1989). The supernatants as well as

high Mw fractions contained a protein with an approximate Mw of 65kD that reacted with the antiserum to human IL-1 beta. These same samples also contained a protein of approximately 70kD that reacted with antiserum to human IL-1 alpha. Also present in the supernatant and in the low Mw fractions were proteins with a Mw of approximately 24kD and 14kD that reacted with anti-IL-1 alpha and anti-IL-1 beta, respectively. Supernatants from mouse P338D1 cells showed four bands of reactivity with antiserum to mouse rIL-1 alpha. These bands were seen in the high Mw fractions with activity for mouse cells.

In summary, catfish IL-1-like molecules include a low Mw form with activity for mouse cells and a high Mw form which is active on catfish cells. Within these two size classifications, there also appear to be two antigenically distinct forms of IL-1: those that react with antibodies to human IL-1 alpha and those that react with antibodies to human IL-1 beta. Northern analysis using cDNA probes for mouse rIL-1 alpha does not detect a RNA species large enough to account for the high Mw form of catfish IL-1. However, Ellsaesser (1989) points out that it is possible that a smaller IL-1 protein is made in channel catfish that subsequently exists as a polymer, or is covalently attached to another undetermined protein. This form of IL-1 is necessary for stimulation of channel catfish cells, yet is inactive on mouse cells. The monomer, or unassociated protein, is active on mouse cells, but not catfish cells. IL-1 from P388D1 (a mouse macrophage cell line) cells appears to be either noncovalently bound together or to form mixed polymers with other proteins, resulting in an active form (for fish cells) which elutes as a large protein by gel filtration but dissociates into smaller components on SDS-PAGE analysis.

Since Ellsaesser (1989) and Clem et al. (1991) reported that only the high Mw mouse IL-1 was active on catfish cells, it is not surprising that the lower molecular weight recombinant human or mouse IL-1 were incapable of stimulating catfish cells. The observation, however, is in apparent opposition to the observations of Hamby and coworkers (Hamby et al., 1986; Sigel et al., 1986) who

reported that a purified protein preparation of human IL-1 with a Mw of 15kD did stimulate channel catfish at suboptimal concentrations of Con A. However, the catfish leukocytes used in this study included monocytes that themselves could have been a source of endogenous IL-1 (Ellsaesser, 1989; Clem et al., 1991).

Interleukin-2 in piscine immune systems. PHA or alloantigen-stimulated pronephric carp leukocytes produce factors in supernatants that are mitogenic for mitogen or alloantigen-induced putative T lymphoblasts, but not resting lymphocytes (Grondel and Harmsen, 1984). This T cell growth activity was partially reduced following adsorption of the supernatant with the mitogen-activated blasts, but not with resting leukocytes. Caspi and Avtalion (1984) have reported data which suggests that only activated cells express receptors for carp TCGF. These researchers have shown that IL-2 rich supernatants from the mouse EL-4 cell line do not support growth of the fish blasts. Clem et al. (1991) also report data which indicate that culture supernatants from catfish T cell lines that are costimulatory for catfish T and B cells do not appear to function in mouse bioassays for IL-2 and IL-4. In addition, recombinant mouse IL-2 and IL-4 do not exhibit *in vitro* functional activity on catfish cells (Clem et al., 1991). However, Caspi and Avtalion (1984) reported that fish T cell blasts and alloreactive MLR-induced fish T cell lines do grow in cultures containing supernatants from either Con A treated rat lymphocytes or PHA-pulsed human peripheral blood cells. Partially purified human IL-2 did not support growth of fish blasts as well as the crude supernatants from which the mitogens had not been removed. Although the lectin concentrations in these crude supernatants were submitogenic for carp lymphocytes, they could have been costimulatory with IL-1 (in the supernatants), thereby evoking the production of homologous IL-2 from the fish cell cultures. The effect of multiple cytokines, which are frequently present in crude supernatants, in many biological assays has yet to be determined in piscine studies.

CHAPTER 2

Requisite signals for B cell antibody production to a T-independent antigen.

Henry W. Ortega and Stephen L. Kaattari [1,2]

Department of Microbiology and Laboratory for Fish Disease Research
Oregon State University
Corvallis, OR 97331-3804

[1] To whom correspondence should be addressed
[2] Current address:

Department of Environmental Science
Virginia Institute of Marine Science
College of William and Mary
Gloucester Point, VA 23062

Abstract

The ability of different subpopulations of rainbow trout (*O. mykiss*) peripheral blood leukocytes to respond to the T-independent antigen trinitrophenylated-lipopolysaccharide (TNP-LPS) was assessed by using an *in vitro* passive hemolytic plaque assay. It is demonstrated that macrophages are required for the provision of accessory function during the antibody response to this antigen. Establishment of this accessory function was demonstrated by the fact that macrophages (adherent leukocytes) were able to restore the capacity for antibody production to isolated lymphocytes (non-adherent leukocytes). Furthermore, supernatants from antigen-stimulated macrophages were sufficient to restore lymphocyte function. Finally limiting dilution analysis (LDA) confirmed this requisite role of the macrophage-derived factor, revealed that the target of this factor(s) is the B cell precursor, and suggests that there may be differential sensitivity of B cell precursors to this factor. Based on the factor source, function, and molecular weight it is suggested that IL-1 is the requisite accessory factor.

Introduction

Resolution of the precise cellular requirements for piscine B cell induction is most easily obtained by *in vitro* analysis. Much of the pioneering work in this area has been accomplished by Clem and coworkers (1991) using the channel catfish (*Ictalurus punctatus*). Cellular fractionation techniques are critical to the resolution of functionally distinct yet cooperative leukocyte subpopulations. Utilization of monoclonal antibodies has permitted the isolation of both surface immunolglobulin positive (sIg^+) and negative (sIg^-) lymphocytes (DeLuca et al., 1983). Together with specific procedures for the removal or isolation of adherent cells (Secombes, 1990), these techniques have been used to delineate many of the requisite cellular associations for catfish B cell induction. To date, many functions of piscine immune systems show similarities to those found in mammals. For example, it has been reported that catfish B lymphocytes (sIg^+) require interaction with both sIg^- lymphocytes (T cells) and macrophages or monocytes in order to generate an antibody response to T cell dependent (TD) antigens (Miller et al., 1985). This TD response also initially requires processing and presentation of the antigen by macrophages (Vallejo et al., 1990). In contrast to this, B cell responses to T cell independent (TI) antigens appear to only require the presence of interleukin 1 (IL-1), with no processing or presentation needed (Miller et al., 1985; Clem et al., 1985).

Recent advances in piscine lymphocyte culture systems permit the use of limiting dilution analysis (LDA) as an alternative method for the assessment of cellular cooperation between lymphocytes in response to antigen (Tripp, 1988; Arkoosh and Kaattari, 1991). This method allows for the elucidation of the sequence of B cell maturation and antigen induced differentiation associated with TI B cell responses at both early and late phases of this response. The more conventional methods used to evaluate *in vitro* piscine B

lymphocyte responses, such as proliferative responses to mitogens or anti-hapten plaque forming cell (PFC) responses, typically resolve only early or late phases of B cell induction by antigen, not both. Limiting dilution analysis has been used in mammalian systems to determine the requirements of many immune responses, as well as to determine the size and frequency of different B cell precursor populations which are to different antigens (Chen et al., 1992). However, in lower vertebrates the utilization of LDA techniques in immunological studies had not been reported until recently. Piscine LDA studies conducted with coho salmon (*Oncorhyncus kisutch*) leukocytes showed that anti-hapten TI PFC responses required a cell type in addition to the B cell for the generation of an optimal response. The assumption was made that, as in both catfish and murine systems, macrophages may be required to assist the B lymphocyte in response to a TI antigen. Subsequently it was shown this cell was adherent and displayed macrophage-like characteristics (Tripp, 1988).

It is reported here that cell free supernatants generated *in vitro* from trout leukocyte cultures contain a factor which can replace adherent cell function in response to the TI antigen TNP-LPS. To identify the requisite stimulus of this factor non-haptenated LPS was used as well as TNP-LPS to generate supernatants. This factor is secreted by the adherent monocyte macrophage cell fraction from trout peripheral blood leukocytes upon stimulation with LPS.

Materials and Methods

Animals. Rainbow trout (*Oncorhynchus mykiss*, Shasta strain) were obtained from the Marine/Freshwater Biomedical Sciences Center at Oregon State University, and maintained at the Salmon Disease Laboratory in Corvallis, OR. This facility receives fish pathogen-free water at a constant temperature of 12°C. Fish weight ranged from 300g - 500g. Fish were fed Oregon Moist Pellet (OMP) commercial salmon food daily.

Mitogen and antigen stock preparation. Stock solutions of *E. coli* 055.B5 lipopolysaccharide (LPS) (Sigma Chemical Company, St. Louis, MO) were diluted in RPMI 1640 to a concentration of 10 mg/ml and pasteurized by incubation in a 70°C water bath for 1 hour.

Trinitrophenylated-lipopolysaccharide (TNP-LPS) was prepared as described previously (Jacobs and Morrison, 1975). TNP-LPS was pasteurized for 45 minutes in a 70°C water bath and stored at 4°C.

Cell preparation and tissue culture. Peripheral blood samples were obtained from fish sacrificed by anesthetic overdose (Kaattari and Irwin, 1985) in benzocaine (ethyl p-aminobenzoate, Sigma). Leukocytes were isolated from peripheral blood by using Ficoll-histopaque (Sigma) separation techniques and cultured in tissue culture medium (TCM) consisting of RPMI 1640 (Gibco, Grand Island, NY) with sodium bicarbonate supplemented with 2% autologous trout plasma and 0.05 mg/ml gentamycin sulfate (Whittaker Bioproducts Inc., Walkersville, MD) as previously described (DeKoning and Kaattari, 1991).

Peripheral blood leukocytes (PBL) were fractionated into lymphocyte and macrophage populations by adherence to plastic as described previously (Secombes, 1990), with minor modifications. Briefly, $3x10^7$ to $6x10^7$ blood or kidney leukocytes were added to

each well of 6 well, flat bottom tissue culture plates (Corning Glass Works, Corning, NY). All wells were pretreated at 17°C overnight with 3 ml of a 10 μg/ml solution of bovine fibronectin (Sigma) in sterile water. Leukocytes were incubated for at least two hours at 17°C in a blood-gas environment. After this incubation the non-adherent cells were gently removed from the plates by aspiration with a Pasteur pipette and transferred to new fibronectin-coated plates and incubated for an additional two hours at 17°C. The remaining adherent cells were washed three times with unsupplemented RPMI 1640 to remove any contaminating non-adherent cells. The adherent macrophage cells were removed from tissue culture plates by gentle scraping with a rubber scraper (Becton Dickinson, Lincoln Park, NJ). Lymphocyte (adherent-depleted) fractions were derived from only the final non-adherent fraction of leukocytes produced from three sequential adherence fractionation steps.

Individual leukocyte fractions were tested for both non-specific leukocyte esterase (Sigma) and sIg staining using a monoclonal anti-trout Ig (DeLuca et al., 1983). Characteristics of PB leukocyte fractions are listed in Table 2.1. In this report, because of the reported staining characteristics and for the sake of convenience, the adherent leukocytes are referred to as macrophages and non-adherent leukocytes are referred to as lymphocytes.

Generation of supernatants. Supernatants were generated by culturing PBL in tissue culture medium (described above) at $2x10^7$ cells/ml. Cells were cultured with 0.4 μg/ml TNP-LPS or LPS for two days, after which time the cells were washed three times with unsupplemented RPMI 1640 to remove any residual TNP-LPS or LPS. The cells were then resuspended in tissue culture medium at a density of $2x10^7$ cells/ml and cultured for an additional four to five days, at which time the culture supernatants were harvested by removal from tissue culture plates. To ensure the removal of cells and debris, supernatants were centrifuged at 1000 x g for 10

minutes and carefully removed from the cell/debris pellet. Supernatants were aliquoted and stored at -20°C for later use.

Supernatants were generated from cell cultures heterologous to those cell cultures being tested, except where noted. All supernatants were used at 50% by volume and added to cells upon initiation of cultures unless otherwise noted.

Plaque forming cell assay. On the day of harvest, 96 well culture plates were centrifuged for 5 minutes at 500 x g. The culture supernatants were removed and cells were resuspended with 200 μl unsupplemented RPMI 1640 per well. Cells secreting anti-TNP antibody were then enumerated by Cunningham plaque assay as previously described (Kaattari and Yui, 1987).

Limiting dilution analysis. Limiting dilution analysis was performed as described previously (Tripp, 1988; Arkoosh, 1990) with little modification. Briefly, responder cells were diluted to the appropriate concentrations with the filler cells thus maintaining a constant concentration of $2x10^7$ cells/ml. In these assays, a standard requirement for the filler cell population is that they must not be able to produce antibodies. To ensure this, filler cells were generated by exposing leukocytes to 6000 rads of ^{60}Co radiation in the low flux region of a gamma irradiator. Aliquots of 10 μl were then distributed to each well of 60 well microculture plates (Intermountain Scientific Corporation, Bountiful, UT) containing irradiated adherent leukocytes (3000 rads) as a source of accessory cells. Tissue culture medium was used in all LDA cultures. For each frequency determination, at least 3 different responder concentrations were assayed with a minimum of 60 replicate cultures per concentration. Negative standards were defined in each experiment by a set of 60 cultures receiving only filler cells plus accessory cells and TNP-LPS. Cells from individual wells were harvested for determination of plaque-forming cells on day 7 of culture. The LDA data were analyzed according to methods previously described (Lefkovits and Waldman, 1979).

Determination of the molecular weight of accessory factor. Molecular sieve column chromatography was used to isolate the active fraction(s) of supernatants. The inert carrier, Sephadex G-100 (Pharmacia, Upsula, Sweden) was prepared as described previously by Tripp (1988). The column (30 ml bed volume) was calibrated using four distinct molecular weight marker proteins (Sigma): alpha-lactalbumin (14.2kD), carbonic anhydrase (29kD), ovalbumin (45kD), and bovine serum albumin (66kD monomer). Upon calibration, 3 ml of Centriprep 3 (Amicon, Inc., Beverly, MA) concentrated supernatant was applied and fractions were collected in the elution buffer pH 7.3 consisting of 100mM sodium chloride and 20mM sodium phosphate (dibasic, anhydrous) in distilled water. Upon elution, fractions were concentrated again, then dialyzed overnight at 4°C against one liter of RPMI 1640 using Spectra/Por 1 cellulose membranes (Spectrum, Houston, TX). Fractions were then filter sterilized and tested for activity as measured by the augmentation of lymphocyte PFC responses.

Results

Requirement for macrophage (adherent leukocytes) in the generation of antibody responses. Isolated lymphocyte (non-adherent) and monocyte/macrophage (adherent) populations demonstrated greatly impaired ability to generate antibodies in response to TNP-LPS (Figure 2.1). This impairment was not due to damage incurred during the fractionation procedure as mixing (1:1) of the two populations resulted in the complete restoration of the antibody response. The residual PFC response (approximately 100 PFC/10^6 cells) in fractionated populations was likely due to contamination of macrophages by lymphocytes in these cultures (Table 2.1).

As this response was induced by a T-independent antigen, it seemed likely that macrophages may be required for accessory function by the B lymphocyte. One such function would be the expression of a requisite soluble factor or interleukin. Supernatants were thus procured by antigenic stimulation of whole leukocytes. These supernatants were added to fractionated and unfractionated leukocytes. Addition of supernatant from stimulated leukocytes completely restored the PFC response to lymphocytes, while effecting no enhancement of the marginal PFC response from macrophages (Figure 2.2). These results, representative of 10 separate experiments, indicate that supernatants generated from leukocyte cell cultures contain a factor(s) which can replace the accessory role of macrophages required for TI anti-hapten PFC responses.

Macrophage-derived cell-free supernatants can restore lymphocyte antibody responses. In order to determine which leukocyte cell fraction produces the soluble factor(s) responsible for the restored response observed in the above experiments, supernatants were generated from macrophage and lymphocyte fractions cultured

separately. These supernatants were then assayed for activity in lymphocyte cultures for their ability to provide the required accessory function in response to antigen. Results from one experiment, representative of seven experiments performed, are shown in figure 2.3. Responses from lymphocytes cultured with supernatants generated from the lymphocyte cultures were equivalent to negative control cultures containing no supernatants. Lymphocyte cultures receiving macrophage-derived supernatants exhibited significantly higher anti-hapten PFC responses than the corresponding negative control cultures. Supernatants generated from macrophage cultures consistently augmented lymphocyte cultures greater than any other fraction tested. This indicates that macrophage-derived cell-free supernatants contain a factor(s) which can provide the accessory function that is required for generating the antibody response to TNP-LPS.

Limiting dilution analysis (LDA) reveals a mode of action of supernatant "factor(s)". Determination of the mode of action of the supernatant "factor" on rainbow trout lymphocytes was achieved by the use of LDA. This analysis can be used to dissect antibody responses into early and late phases. Analysis of both unfractioned leukocyte and lymphocyte cultures revealed that the conditions used were not limiting for one cell type (Figures 2.4 and 2.5). However, addition of a small aliquot of macrophages to each well generated a curve indicative of single hit kinetics (Figure 2.4). Therefore, it is apparent that trout B lymphocytes require a macrophage-derived accessory functions in order to generate an optimal PFC response to TNP-LPS *in vitro*. To test if supernatants can provide these required accessory function(s) generated from macrophages LDA was performed using lymphocyte cultures supplemented with these supernatants. As shown in figures 2.4 and 2.5, single hit kinetics (linear plot) can be generated in LDA cultures by the simple addition of macrophage-derived supernatants. This experiment also indicates that cell to cell contact between B cells and accessory cells is not an absolutely required accessory function.

Titration of this macrophage-derived supernatant in limiting dilution analysis cultures resulted in an increase in the lymphocyte precursor frequency (Figure 2.5). This suggests that the accessory factor effects lymphocytes at the early stages of PFC responses. Moreover, the mechanism of this effect is to increase the numbers of pre-B cells available to respond to antigenic stimulus.

Determination of the accessory factor molecular weight. Determining the molecular weight of the accessory factor was acheived by the use of molecular sieve column chromatography. The resulting eluates were tested for accessory activity as measured by the ability to augment lymphocyte PFC responses. As shown in figure 2.6, the active fraction in macrophage-derived supernatants elute at a molecular weight range from 19-24kD. No other fractions had accessory activity in these supernatants.

LPS induces IL-1-like factor production. In order to ascertain whether the LPS signal alone is sufficient for the elaboration of IL-1-like activity, 0.4μg/ml LPS was compared to 0.4μg/ml TNP-LPS. In the absence of the hapten (TNP) on TNP-LPS, the induction of this factor can be verified, as shown in figure 2.7. Stimulation by 0.4μg/ml TNP-LPS or 0.4μg/ml LPS results in the generation of supernatants with similar accessory activities. This verifies LPS as the inducing stimulus of the IL-1-like factor.

Discussion

It is shown here that adherent, esterase-positive, sIg^- leukocytes (monocytes/macrophages) are required for accessory function in the generation of antibody to a T-independent (TI) antigen, TNP-LPS. Most importantly it was shown that the mechanism of this macrophage accessory function does not require direct cell to cell contact. Previous studies in both mammalian and other piscine systems have shown that cell-free supernatants generated by LPS-stimulated macrophages supply accessory cell function for TI PFC responses of cultures depleted of macrophages (Miller et al., 1985; Clem et al., 1985). Establishment of monokine secretion as the mode of accessory cell function in trout was achieved by employment of cell fractionation techniques. Macrophages were separated from rainbow trout peripheral blood leukocyte suspensions using adherence and multiple passages onto fibronectin-coated surfaces before cultured in the presence of TNP-LPS. These macrophage-derived cell-free supernatants were then assayed for the ability to replace macrophages in the induction of *in vitro* antibody responses. In addition, non-haptenated LPS was used to stimulate cells in order to verify the requisite stimulus for producing this factor. The lymphocyte antibody response was restored upon the addition of TNP-LPS- or LPS-derived supernatants. Therefore, at least one factor of accessory cell function in rainbow trout is the production of a biologically active monokine(s). Molecular weight fractionation studies indicate that this monokine has an approximate molecular weight of 19-24kD (Figure 2.6). Because of the accessory activity, macrophage source, and molecular weight data we suggest that the predominantly active monokine in these supernatants is an IL-1-like factor.

The LDA confirms and further elucidates the mode of action of the monokine particularly when coupled with cell fractionation techniques. The *in vitro* form of LDA utilizes microculture

techniques to permit isolated development of functional clones of B lymphocytes from a single precursor (Lefkovits and Waldman, 1979). Thus LDA results in the quantitative analysis of B cell precursor frequency and clone size, as well as the determination of cellular cooperativity. In this study the requisite for more than one cell type was indicated by the non-linear kinetics exhibited by lymphocyte cultures in response to TNP-LPS (Figures 2.4 and 2.5). Linear kinetics occurred when the lymphocyte fraction was coincubated in a saturating concentration of either macrophages or macrophage-derived supernatant (Figure 2.4). Thus, as supernatant is sufficient for the generation of linear kinetics, actual physical contact between the macrophage and B lymphocyte is not required. These findings are comparable to the accessory role of macrophages observed in the murine system, wherein MHC-II$^-$ (negative) IL-1 producing adherent cells are fully capable of supplying accessory function, whereas MHC-II$^+$ (positive) non-IL-1 producing cells cannot do so (Sinha et al., 1987). In mammalian systems it has also been reported that IL-1 has a direct role in B cell activation and IL-1 secretion is the only accessory cell function required for TI B cell activation (Yamaga et al., 1978).

The utilization of limiting dilution analysis for the study of lower vertebrate immune responses has not been reported until recently (Tripp, 1988; Arkoosh and Kaattari, 1991). These initial limiting dilution studies revealed that upon dilution of unfractioned leukocyte populations multi-hit kinetics were observed indicating that minimally two cell types were required for generating a positive response. When saturating concentrations of macrophages were added to LDA cultures the comparable dilution of whole leukocytes yielded single-hit kinetics. In the study reported here it is shown that restoration of at least one macrophage accessory cell function can be acheived by the addition of supernatants generated by LPS stimulation of leukocyte as well as macrophage cell cultures. Column chromatography fractionation of these supernatants using a molecular sieve column revealed a biologically active peak eluting at 19-24kD (Figure 2.6). Based on these findings and those previously

reported in both mammalian and piscine systems it is proposed that IL-1 or an IL-1-like factor is the monokine required for trout accessory function to TI antigens.

The fact that cytokines play an important immunoregulatory role in piscine systems has been demonstrated by several investigators. The action of cytokines on both specific and non-specific immune cells in fish has been the focus of several reports. Rainbow trout lymphocytes have been shown to secrete a factor which directly affects macrophages (Secombes, 1987; Graham and Secombes, 1990a; Graham and Secombes, 1990b). Catfish macrophages have been shown to secrete a cytokine that serves an accessory function in TD anti-hapten antibody responses (Clem et al., 1985). It has also been reported that stimulation of catfish long term monocyte cultures with LPS induces secretion of high and low molecular weight species of IL-1 that are biologically active for catfish as well as mouse T cells (Vallejo et al., 1991). A biologically active invertebrate cytokine which can be blocked with antibodies to mammalian IL-1 alpha has been extracted from starfish coelomocytes (Burke and Watkins, 1991), and invertebrate hemocytes have been shown to react to human TNF and IL-1 (Hughes et al., 1991). Given this information it is likely that the LPS stimulated rainbow trout macrophages in our study are secreting at least one IL-1-like monokine which can restore the TI PFC responses of lymphocyte cultures. Although very likely, it has yet to be determined if more than one monokine is being produced and secreted by these macrophages.

It is interesting to note the increase in B cell precursor frequency in lymphocyte cultures supplemented with increasing concentrations of leukocyte derived supernatants (Figure 2.5). One explanation for this is that there are subpopulations of B lymphocytes with differing sensitivities to IL-1. Therefore there is a lower frequency observed when the concentration of supernatant is only 50%, but much greater when 90%. A sensitive population is triggered by the relatively low dose of supernatant while the sensitive and relatively insensitive population are triggered by the

high dose of supernatant. Another possible explanation is that when the concentration of IL-1 in the culture medium is very low the B lymphocyte can only be activated when in close proximity to an activated, IL-1 secreting macrophage. Only the antigen-activated B precursors that are proximal to IL-1 secreting macrophages will be able to accrue enough IL-1 to differentiate into antibody producing cells. However, if an IL-1 containing supernatant is supplied, then the concentration of IL-1 may be sufficiently high so as to permit a greater percentage of B lymphocyte precursors to differentiate. In this scheme, no presentation or processing of the TI antigen is required, since no cell-cell contact is required. Another implication of this data is that the antigen itself must activate the B lymphocyte to a stage of IL-1 receptive differentiation. At this stage provision of IL-1 completes the differentiative sequence leading to the production of antibody. Whether antigen can provide an initial first activation step for TD antigens has yet to be addressed. A model based upon the above studies which describes piscine B cell responses to TI antigens has been reported recently (Kaattari, 1992).

These studies indicate that rigorous macrophage cell depletion methods must be used to demonstrate this fact. Sizemore et al. (1984) have shown that nonadherent leukocyte cultures containing as little as 1% macrophages permit mitogenic responses to both Con A and LPS that were nearly indistinguishable from those of unfractionated cells. In our studies, depletion of adherent macrophages by multiple passages onto fibronectin-coated surfaces was found to be an efficient and simple method. Although the adherence method used in this study is quick and convenient for the removal of most macrophages, ostensibly some B cells appear to be present in the adherent cell or macrophage fraction thus a limited PFC response was elicited. It is also likely that some macrophages were not removed from the macrophage depleted or non-adherent population using the adherence technique and provided limited accessory cell function for a minimal PFC response (Figures 2.1 and 2.2).

In conclusion, based on previous studies and the findings reported here it is becoming increasingly apparent that the induction of humoral immune reactions are mediated through macrophage functions similar to those observed in higher vertebrates. Further investigation and elucidation of the function of macrophages and macrophage-derived soluble mediators in various piscine and invertebrate systems may reveal this similarity to higher vertebrates as well as further support the use of piscine immune systems as simple and useful immunological models for higher vertebrates.

Acknowledgements

The authors wish to thank Dr. M. Adkison, J. Piganelli, J. Hansen, and P. Wood for their critical review of this manuscript, and J. Bishop and L. Gilkey for technical assistance. This work was supported through the Oregon Sea Grant Award No. R/Aq-60. Agricultural Experiment Station Technical Paper No. 10312.

Leukocyte cell fraction	% surface Ig+	% non-specific esterase+
Unfractionated	51	18
Adherent	2	96
Non-adherent	62	3

Table 2.1: **Characteristics of adherence fractionated leukocyte populations.** The percentage of cells expressing surface immunoglobulin (sIg+) was evaluated by immunofluorescent staining methods described previously (DeLuca et al., 1983). Non-specific esterase tests were performed as indicated by the manufacturers instructions (Sigma, alpha-Napthyl Acetate Esterase kit).

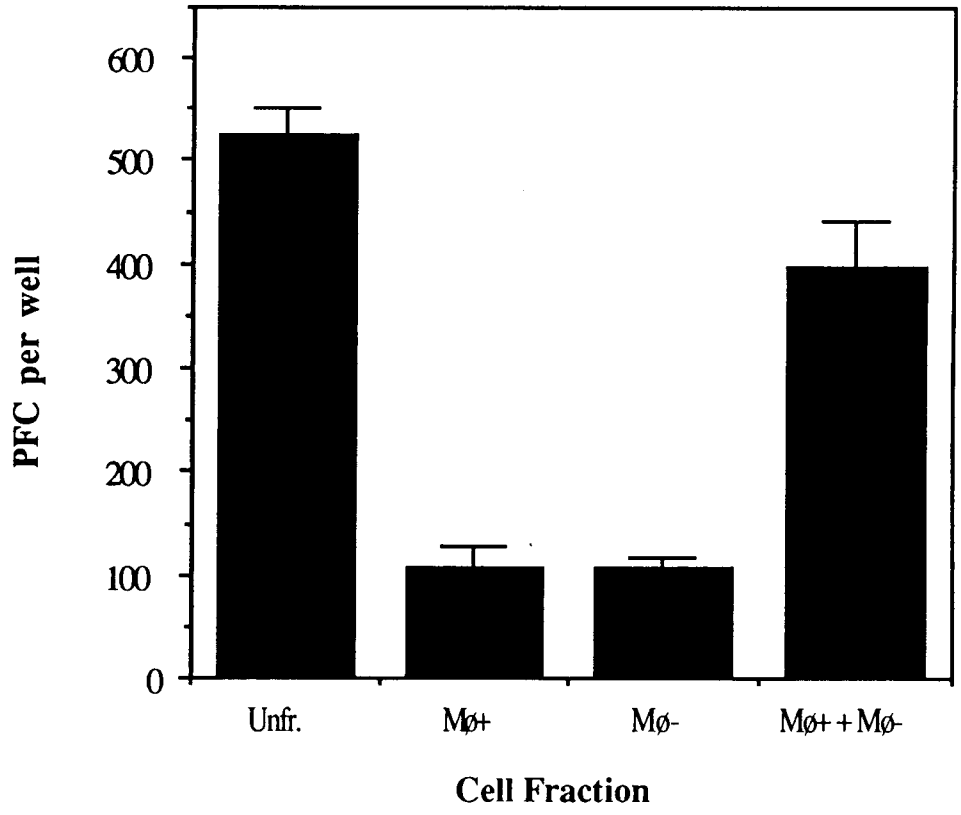


Figure 2.1: ***In vitro* antibody responses of adherence fractionated trout PBL.** Unfractionated (Unfr.), adherent ($M\phi^+$), non-adherent or lymphocytic ($M\phi^-$), and re-mixed ($M\phi^+ + M\phi^-$) leukocytes were cultured at a concentration of 10^7 cells/ml in 100μl tissue culture medium containing 0.4μg/ml TNP-LPS. All cultures were assayed for anti-TNP antibodies on day 9. The number of PFCs per 10^6 cells were plotted for each corresponding fraction of leukocytes cultured. Each bar represents the mean of 3 cultures containing 10^6 leukocytes each and the error bar represents 1 standard deviation from the mean.

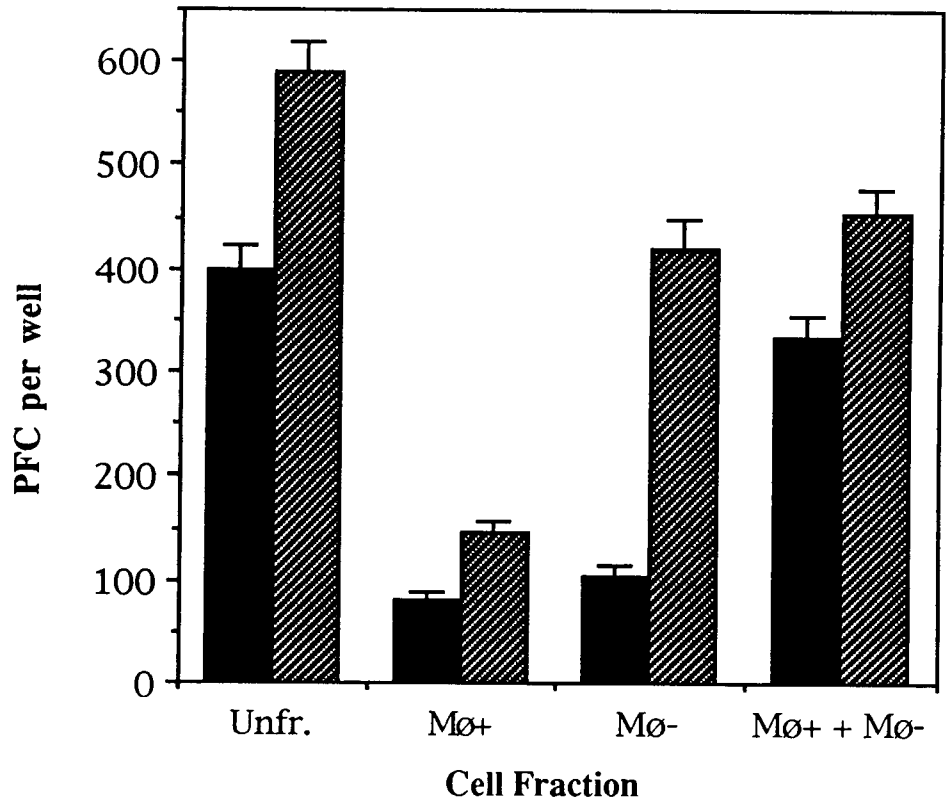


Figure 2.2: ***In vitro* antibody responses of adherence fractionated PBL supplemented with supernatants.** Cells were cultured at 10^7 cells/ml in 100μl tissue culture medium containing 0.4μg/ml TNP-LPS supplemented with 50% tissue culture medium (■) or 50% leukocyte derived supernatants (▨) by volume. Supplements were added to cultures upon initiation and all cultures were harvested on day 9. The number of PFCs per 10^6 cells were plotted for each corresponding fraction of leukocytes cultured. Each bar represents the mean of 3 cultures containing 10^6 lymphocytes each and the error bar represents 1 standard deviation from the mean.

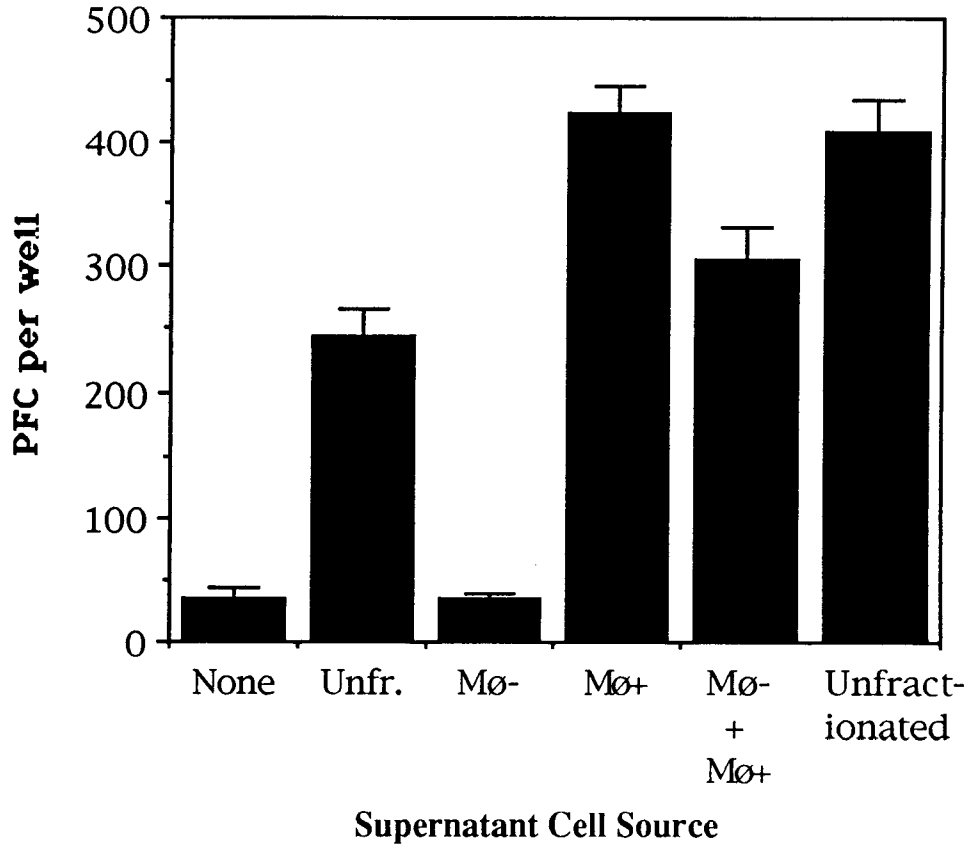


Figure 2.3: **The effect of supernatants generated from individually fractionated and cultured leukocytes on antibody production.** Supernatants were generated from LPS stimulated unfractioned (Unfr.), macrophage (Mø+), macrophage depleted (Mø-), and macrophage depleted leukocytes supplemented with macrophages (Mø- + Mø+) cultures. Lymphocytes were cultured at 10^7cells/ml in 100μl tissue culture medium containing 0.4μg/ml TNP-LPS plus 50% of each supernatant (by volume). Control cultures containing unfractionated leukocytes were assayed for comparison. The number of PFCs generated were plotted for each corresponding culture. Each bar represents the mean of 3 cultures and the error bar represents 1 standard deviation from the mean.

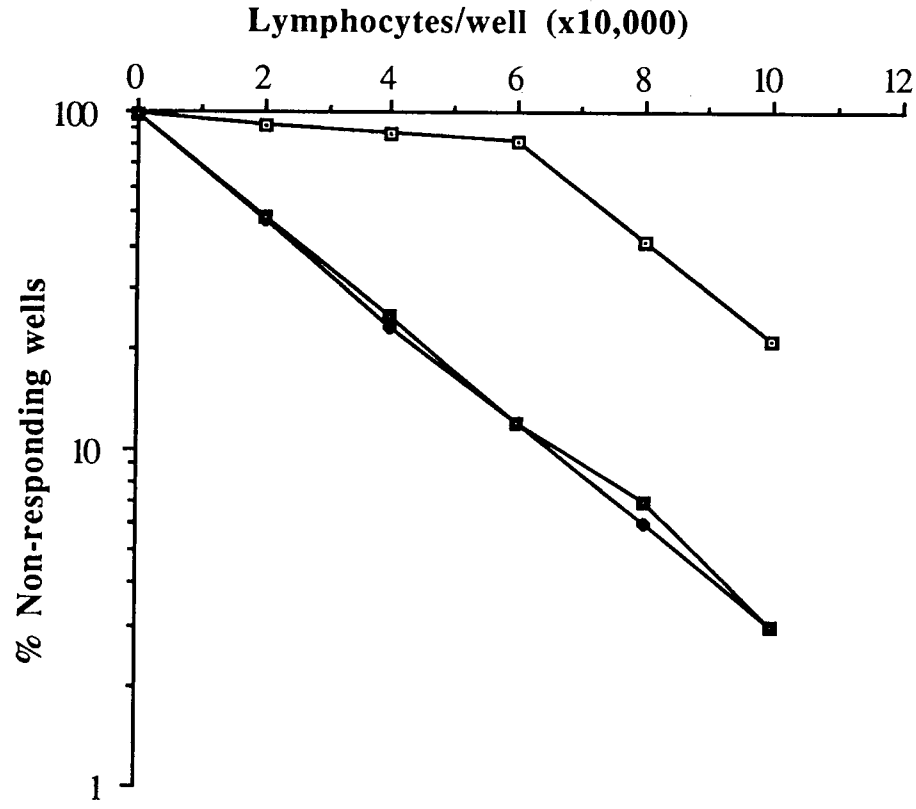


Figure 2.4: **Limiting dilution analysis of lymphocytes cultured with macrophages or macrophage-derived supernatants.** Lymphocytes were cultured alone (—□—) or in the presence of either macrophages (—●—) or 50% macrophage-derived supernatant (—■—). All lymphocytes were cultured in tissue culture medium containing 0.4μg/ml TNP-LPS. The supernatant was added upon culture initiation. All cells were harvested on day 7 of culture and the fraction of non-responding wells were plotted for each corresponding number of responding lymphocytes per well. Sixty replicate cultures were analyzed for each responder cell concentration.

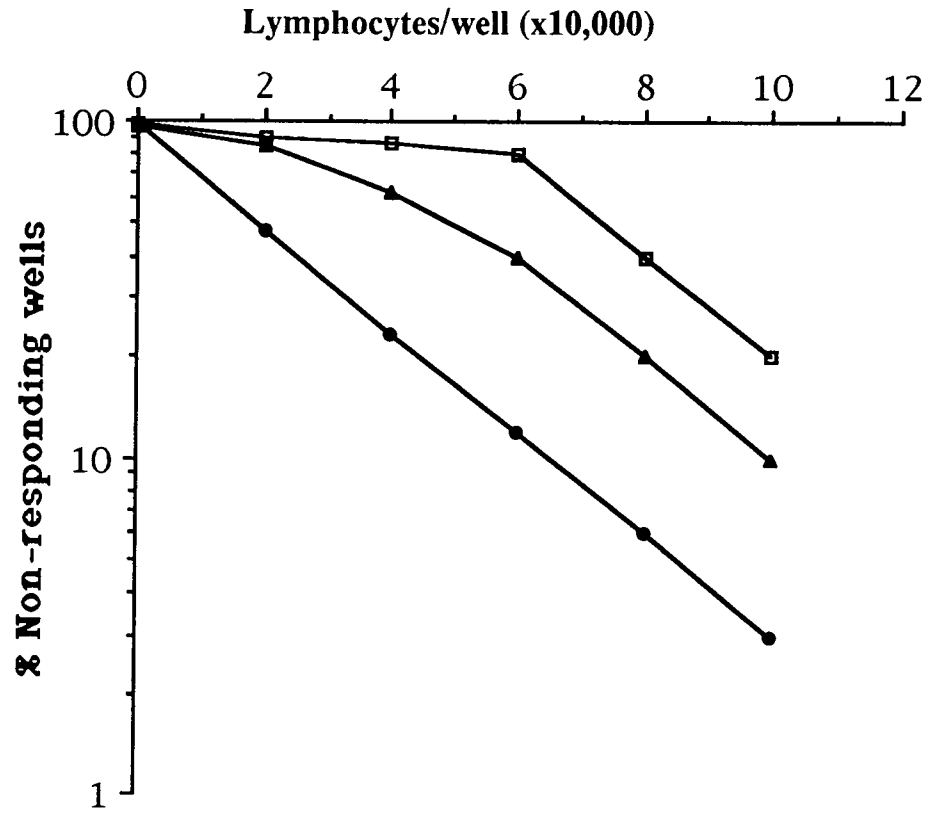


Figure 2.5: **Limiting dilution analysis of lymphocyte cultures supplemented with supernatants.** Lymphocytes were cultured at 10^7 cells/ml in tissue culture medium containing 0.4μg/ml TNP-LPS supplemented with either 0% (▫), 50% (▲), or 90% (●) supernatants by volume. Supernatants were added to cultures upon initiation. Cells were harvested on day 7 of culture and the fraction of non-responding wells were plotted for each corresponding number of responding lymphocytes per well. A minimum of 60 replicate cultures were analyzed for each responder cell concentration.

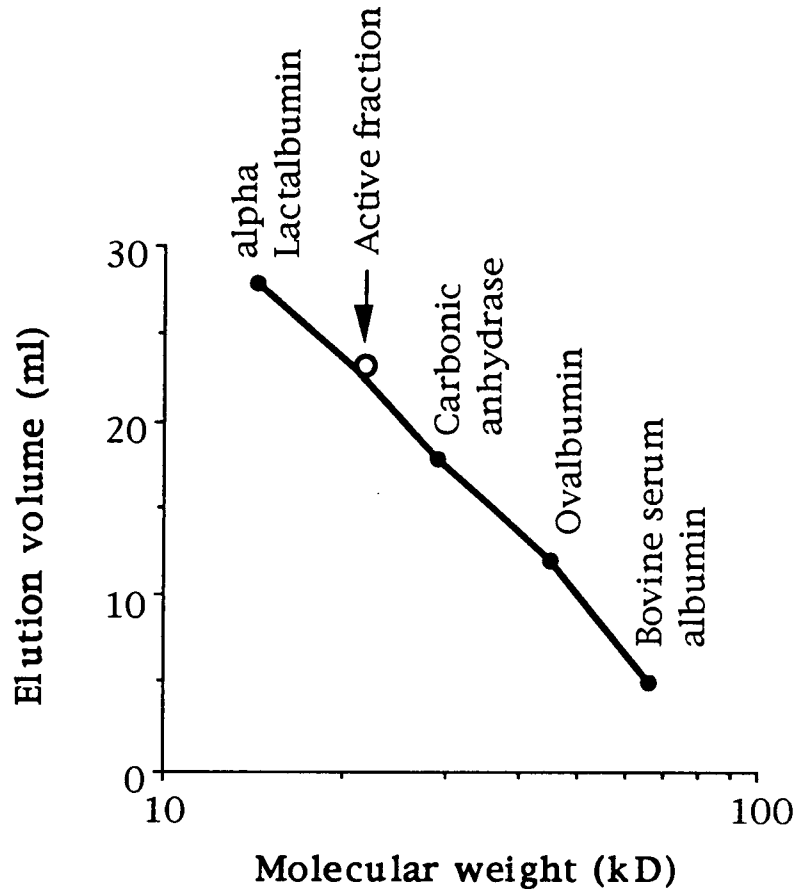


Figure 2.6: **The elution profile for accessory activity in chromatographed macrophage-derived supernatants.** Solid circles represent the elution profiles of protein molecular weight markers: alpha-lactalbumin (14.2kD), carbonic anhydrase (29kD), chicken egg albumin (45kD), and bovine serum albumin (66kD). The open circle represents the molecular weight at which accessory activity was displayed.

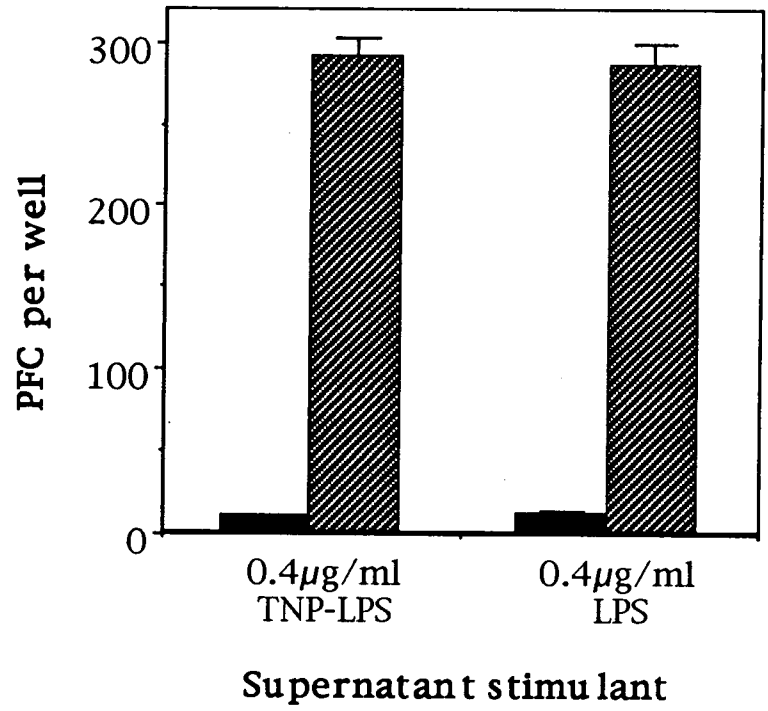


Figure 2.7: **Comparison of TNP-LPS- and LPS-derived supernatant IL-1-like activity.** All supernatants were generated by culturing leukocytes in TCM supplemented with 0.4μg/ml TNP-LPS or 0.4μg/ml LPS for four days. Lymphocytes were cultured at 10^7 cells/ml in TCM supplemented with 50% supernatants by volume in the presence (▨) or absence (■) of antigen. The number of PFCs generated was plotted for each supernatant tested. Each bar represents the mean of three replicate cultures and the error bar represents one standard deviation from the mean.

CHAPTER 3

Induction of cytokines and their receptors during antigen-specific and polyclonal responses.

Henry W. Ortega and Stephen L. Kaattari [1,2]

Department of Microbiology and Laboratory for Fish Disease Research
Oregon State University
Corvallis, OR 97331-3804

[1] To whom correspondence should be addressed
[2] Current address:

Department of Environmental Science
Virginia Institute of Marine Sciences
College of William and Mary
Gloucester Point, VA 23062

Abstract

It has been previously shown that rainbow trout macrophages produce and secrete an IL-1-like monokine which can provide an accessory function required for the generation of an *in vitro* T-independent antibody response. It is shown here that this IL-1-like factor, as measured by the provision of accessory function for antibody responses, is produced as early as 24 hours and attains its highest concentration by day 4 post LPS stimulation. Stimulation with high (200μg/ml) and low (0.2μg/ml) doses of LPS generates supernatants with different activities. These activities include polyclonal activation of lymphocytes in the absence of antigen, as well as an accessory role in antigen specific responses. Stimulation of leukocytes with a low dose of LPS appears to only induce the IL-1-like accessory factor, whereas high doses of LPS induce a factor which polyclonally activates lymphocytes. This difference in activity is also demonstrated by the adsorption of supernatants onto paraformaldehyde-fixed lymphocytes. High dose LPS supernatant activity is reduced following adsorption with either non-activated or antigen-activated lymphocytes, whereas low dose LPS supernatant activity is reduced only when adsorbed with antigen-activated lymphocytes. This demonstrates the presence of at least two separate cytokine factors with distinct biological activities. This also suggests that lymphocyte surface receptors for polyclonal activating factor are present on both antigen- and non-activated lymphocytes, while only activated lymphocytes express a surface receptor for the IL-1-like factor. Finally, the addition of factor to cultures at later stages of the PFC response decreases its efficiency in providing the accessory activity required for anti-hapten PFC responses of lymphocytes.

Introduction

A wide variety of known nonspecific, soluble mediators exist that regulate both the afferent and efferent limbs of the immune response. These include interleukins 1 (IL-1) through 13 (IL-13), each having somewhat distinct but pleiotropic regulatory functions. Interleukin one (IL-1) was first detected in cultures of human peripheral blood adherent cells, and the biological properties originally ascribed to this molecule included the synergistic enhancement of the proliferative responses of murine splenocytes and thymocytes to lectins such as Con A and PHA, as well as the enhancement of antibody production by T cell depleted splenocytes (reviewed by Dinarello, 1990). Presently, IL-1 is known to affect B and T cell proliferation and function (Corbel and Melchers, 1984; Pike and Nossal, 1985; Sinha et al., 1987; Inaba and Steinman, 1986). IL-1 also has been demonstrated to increase the expression of immunolglobulin (Ig) receptors, as well as to promote the expression of differentiation antigens on T cell membranes (Hagicuara et al., 1987; Bonnefoy et al., 1989). It has been demonstrated that IL-1 has a direct role in B cell activation and IL-1 secretion is the only accessory cell function required in T independent (TI) B cell activation (Sinha et al., 1987). Induction of T dependent (TD) B cell responses are also dependent on the elaboration of IL-1 by accessory cells (reviewed by Unanue, 1984). Thus it is clear that IL-1 is an important accessory factor in both TI and TD B cell responses.

Stimulation of catfish monocyte cell lines with LPS induces secretion of relatively high levels of high and low molecular weight species of IL-1 that are active on both catfish and mouse T cell lines (Vallejo et al., 1991). It has also been shown that *in vitro* activation of fish primary macrophage cultures induces the elaboration of IL-1, which appears to be required for both TI and TD B cell responses (Clem et al., 1985). In addition, interferon and macrophage-

activating factor have been elicited from salmonid leukocytes. These two distinct cytokines appear to possess comparable macrophage-activating functions as observed in mammals (Graham and Secombes, 1990; Smith and Braun-Nesje, 1982). In the catfish system it has been reported that B cell responses to TI antigens appear only to require the presence of macrophage-derived IL-1 (Clem et al., 1985), with no processing or presentation needed.

To define interleukins which have the capacity to activate B cells and mediate their growth and maturation to antibody-secreting cells, the induction of Ig secretion by B cells or B cell lines is often used (Isakson et al., 1982; Pure et al., 1983). In piscine studies, however, the current lack of B cell lines available hinders the study of interleukins. Subsequently, piscine interleukins have been characterized by measuring the indirect effects of culture supernatants on target primary leukocyte populations *in vitro.*

The goal of this study was to characterize the production and effects of an IL-1-like factor which is secreted by LPS-stimulated trout macrophages. Because of the relatively low yield of purified macrophages in our system, unfractionated leukocytes were used to generate supernatants. Interestingly, stimulation of leukocytes with high (200μg/ml) and low (0.2μg/ml) doses of LPS generated supernatants with different activities. The induction of these factors and their receptors are characterized and discussed in this study.

Materials and Methods

Animals. Rainbow trout (*Oncorhynchus mykiss*, Shasta strain) were obtained from the Marine/Freshwater Biomedical Sciences Center at Oregon State University, and maintained at the Salmon Disease Laboratory in Corvallis, OR. This facility receives fish pathogen-free water at a constant temperature of 12°C. Fish weight ranged from 300g - 800g. Fish were fed Oregon Moist Pellet (OMP) commercial salmon food daily.

Mitogen and antigen stock preparation. Stock solutions of *E. coli* 055.B5 lipopolysaccharide (LPS) (Sigma) were diluted in RPMI 1640 to a concentration of 10 mg/ml and pasteurized by incubation in a 70°C water bath for 1 hour.

Trinitrophenylated-lipopolysaccharide (TNP-LPS) was prepared as described previously (Jacobs and Morrison, 1975). TNP-LPS was pasteurized for 45 minutes in a 70°C water bath and stored at 4°C.

Cell preparation and tissue culture. Peripheral blood samples were obtained from fish sacrificed by anesthetic overdose in benzocaine (ethyl p-aminobenzoate, Sigma Chemical Company, St. Louis, MO). Leukocytes were isolated and cultured in tissue culture medium consisting of RPMI 1640 (Gibco, Grand Island, NY) with sodium bicarbonate supplemented with 2% autologous trout plasma and 0.05mg/ml gentamycin sulfate (Whittaker Bioproducts Inc., Walkersville, MD) as previously described (DeKoning and Kaattari, 1991).

Peripheral blood leukocytes (PBL) were fractionated into lymphocyte and macrophage populations by adherence to plastic as described previously (Secombes, 1990), but with minor modifications. Briefly, 3 to $6x10^7$ blood leukocytes were added to each well of 6 well flat bottom tissue culture plates (Corning Glass

Works, Corning, NY). All wells were pretreated at 17°C overnight with 3 ml of a 10μg/ml solution of bovine fibronectin (Sigma) in sterile water. Leukocytes were incubated for at least two hours at 17°C in a blood-gas environment. After this incubation, the non-adherent cells (lymphocytes) were removed from the plates by aspiration with a Pasteur pipette and transferred to new fibronectin-coated plates, while the remaining adherent cells were washed three times with unsupplemented RPMI 1640 to remove any contaminating lymphocytes. For macrophage/monocyte depleted fractions, only the final non-adherent fraction of lymphocytes produced from 3 sequential fractionation steps was used in experiments to avoid contamination of macrophages. Adherent cells were removed from tissue culture plates by gentle scraping with a rubber scraper (Becton Dickinson, Lincoln Park, NJ). This procedure results in 96% esterase-positive cells in the adherent fractions and 97% lymphocytes in the non-adherent fractions (Ortega and Kaattari, 1993a).

Generation of supernatants. Supernatants were generated by culturing PBL in tissue culture medium containing an appropriate dose of LPS at $2x10^7$ cells/ml. On the appropriate day of harvest, supernatants were removed from cultures and centrifuged at 1000 x g for 10 minutes, then passed through 0.2μm filters (Schleicher and Schuell, Inc., Keene, NH) to remove the remaining LPS in supernatants. Filtered supernatants were stored at -20C° for later use.

Supernatants were generated from cell cultures heterologous to those tested, except where noted. All supernatants were used at 50% by volume and added to cells upon initiation of cultures unless otherwise noted.

Adsorption of supernatant accessory activity by incubation with lymphocytes. Peripheral blood leukocytes and red blood cells (RBC) were cultured at 10^7 cells/ml for 3 days in either tissue culture medium (TCM) supplemented with 0.4μg/ml TNP-LPS (antigen-

activated) or unsupplemented TCM (non-activated). On the third day all cells were washed 3 times with unsupplemented RPMI, and fixed with 4% paraformaldehyde dissolved in phosphate-buffered saline (PBS; pH 7.4) for 2 hours at 4°C. After fixation, the cells were washed once with PBS, then three times with unsupplemented RPMI. Following the final wash 10^8 paraformaldehyde-fixed, antigen- or non-activated cells were resuspended in 0.5 ml of either high (200μg/ml) or low (0.2μg/ml) dose LPS-derived bulk stock supernatants and adsorbed 18 hours at 17°C, after which the adsorbed supernatants were harvested as described above. The accessory activity of adsorbed supernatants was compared to their corresponding unadsorbed supernatant activity by evaluating their ability to augment TNP-LPS induced plaque forming cell (PFC) responses.

Mitogen Assay. Twenty hours prior to harvest, 10μl (1μCi) of ^{3}H-thymidine, specific activity of 6.7 Ci/mmol, (ICN Biomedicals, Inc., Irvine, CA) diluted in RPMI was added to each well. Cells were harvested as previously described by (Kaattari and Yui, 1987). Results are expressed as either counts per minute (cpm) or stimulation indices (cpm of cultures with mitogen/cpm of cultures without mitogen).

Plaque forming cell assay. On the day of harvest, 96 well culture plates were centrifuged for 5 minutes at 500 x g. The culture supernatants were removed and cells were resuspended in 200 μl unsupplemented RPMI per well. Cells secreting anti-TNP antibody were then enumerated by Cunningham plaque assay as previously described (Arkoosh and Kaattari, 1991).

Determination of the molecular weight of cytokine factors. Molecular sieve column chromatography using Sephadex G-100 (Pharmacia, Upsula, Sweden) was prepared as described previously by Tripp (1988). The column (30 ml bed volume) was calibrated using five distinct molecular weight marker proteins (Sigma): alpha-lactalbumin (14.2kD), carbonic anhydrase (29kD), ovalbumin (45kD),

and bovine serum albumin (66kD monomer). Upon calibration, 3 ml of Centriprep 3 (Amicon, Inc., Beverly, MA) LPS-derived supernatant was applied to the column. Fractions were then collected in the elution buffer pH 7.3 consisting of 100mM sodium chloride and 20mM sodium phosphate (dibasic, anhydrous) in distilled water. Upon elution, fractions were reconcentrated then dialyzed overnight at 4°C against one liter of RPMI 1640 using Spectra/Por 1 cellulose membranes (Spectrum, Houston, TX). Fractions were then filter sterilized and the activity of each fraction compared as measured by the augmentation of lymphocyte PFC responses.

Results

Removal of LPS from culture supernatants. In this study LPS was removed from culture supernatants by filtration (0.2μm). This procedure was deemed essential so that the direct effect of LPS would not confound analysis. Verification of filtering efficiency was accomplished by prefiltering TCM containing LPS prior to stimulation of cultures. As shown in figure 3.1, PBL cultures neither proliferate nor produce PFCs when incubated with prefiltered LPS-supplemented TCM, whereas there is a dose-dependent leukocyte response to unfiltered LPS-supplemented TCM. This suggests that LPS can be removed from or reduced to submitogenic levels in TCM by filtering with 0.2μm membranes. Interestingly, upon incubation with supernatants filtered after five day LPS stimulation (post-filtered) PBL both proliferate (Figure 3.1A) and produce PFCs (Figure 3.1B). This indicates that a non-LPS factor can induce both leukocyte proliferation and PFC production in these cultures.

LPS dose response for generation of factor. To determine if the IL-1-like factor can be induced by using high doses of LPS, supernatants were generated using increasing doses of LPS and assayed for accessory activity. Cultures were stimulated with LPS upon initiation and all supernatants were harvested on day 4. Factor activity was measured by the augmentation of antigen specific responses. As shown in figure 3.2, supernatants generated from low doses of LPS stimulation equally restored lymphocyte PFC responses as did 0.4μg/ml TNP-LPS-derived supernatants. In contrast, high dose (200μg/ml) LPS supernatants appear to polyclonally activate lymphocytes. This finding suggests that high and low dose LPS-derived supernatants may have distinct biological activities as measured by their ability to induce polyclonal activation in lymphocyte cultures. Alternatively, high dose LPS stimulation may

generate supernatants with higher levels of the IL-1-like factor which may induce antigen non-specific activation.

Determining the molecular weight of cytokine factors. To verify the presence of a second soluble factor distinct from the IL-1-like factor, high and low dose LPS-derived supernatants were fractionated by molecular weight. Fractionation using molecular sieve column chromatography revealed that all active fractions eluted at a range of 19-24kD (Figure 3.3). This suggests that these supernatants contain either different factors with similar molecular weights, or simply different concentrations of the same IL-1-like factor.

Adsorption of factor activity by lymphocytes. As cytokine actions are largely mediated by receptors, it was hypothesized that trout lymphocytes may express distinct surface receptors for the seemingly distinct active factors in high and low dose LPS-derived supernatants. To test this, replicate lymphocyte cultures were established in which one culture was activated with antigen and one culture was non-activated. After two days, all lymphocytes were paraformaldehyde-fixed, and either high or low dose LPS supernatants were adsorbed with the fixed lymphocytes. Following adsorption, supernatant activity was measured by both the provision of accessory activity in PFC responses and mitogenic stimulation of lymphocytes. As shown in figure 3.4A, high dose LPS-derived supernatants display both reduced accessory activity in lymphocyte PFC responses as well as a reduced ability to polyclonal activate lymphocytes in the absence of antigen following adsorption with antigen-activated or non-activated lymphocytes. In contrast, low dose LPS-derived supernatants (Figure 3.4B) do not polyclonally activate lymphocytes in the absence of antigen, and have reduced accessory activity only when adsorbed with antigen-activated lymphocytes. This suggests that at least two separate factors exist which are induced by stimulating leukocytes with different doses of LPS. Moreover, this suggests that both antigen-activated and non-

activated lymphocytes express surface receptors for a high dose LPS-derived factor, whereas only antigen-activated lymphocytes express receptors for the low dose LPS-derived factor.

Kinetics of generation of factor. To further characterize these high and low dose LPS-induced factors, the kinetics of factor generation was evaluated for each supernatant. The kinetics of generation of these factors from PBL cultures stimulated with LPS was ascertained by stimulating seven replicate cultures with 0.2μg/ml or 200μg/ml LPS. On the appropriate days, supernatants were harvested and filtered in order to remove any residual LPS within supernatants. Supernatant activity was measured by the augmentation of PFC responses of lymphocyte cultures with or without antigen.

As shown in figure 3.5, high and low dose LPS-derived supernatants augment PFC responses when harvested as early as 1 day post LPS stimulation. Optimal activity is observed in supernatants harvested on day 4 or 5 post LPS stimulation and remains at this level through day 7. This indicates that factors are produced and secreted as early as 24 hours post LPS stimulation, and the generation of factors peak by day 4 post stimulation. Addition of polyclonal activating factor (high dose LPS supernatant) to non-antigen activated cultures generates a kinetic response similar to appear to that response observed in lymphocyte cultures stimulated with antigen. In contrast, low dose LPS supernatant IL-1-like factor is produced with slower kinetics, and does not polyclonaly activate lymphocytes (Figure 3.5).

Determining the relative activity of factor present in supernatants. In order to establish when these factors are being produced at optimal levels, the relative activity of supernatants harvested on days 0-7 was evaluated. Upon observation of the kinetics data (Figure 3.5) factor levels in supernatants harvested on days 4-7 appear to be equivalent. But because all supernatants in the kinetics studies were used at 50% (by volume) it was possible that saturating levels of these factors were present in those cultures

supplemented with supernatants harvested after day 4. By titrating these supernatants the relative levels of factor in each supernatant can be more accurately evaluated. High and low dose LPS-derived supernatants were added to lymphocytes cultured in the presence of antigen and assayed for PFC responses on day 9 of culture. Titration of high dose LPS supernatants revealed that the relative units of activity of factor in supernatants markedly increased on days 3-7 (Table 3.1; Figure 3.6A), with maximum concentrations of factor present by days 5 and 6. In contrast, the low dose LPS supernatants showed a marked increase in factor on days 5-7 (Table 3.1; Figure 3.6B). These data confirm that the kinetics of these two factors have distinct patterns, which again indicates that there are at least two separate factors present in these supernatants.

Kinetics of addition of factor to Mø- cultures. In order to determine when factor is required during the TI PFC response to TNP-LPS, supernatants were added to PB lymphocyte cultures on days 0-8. Activity was measured by the restoration of antibody response as measured on day 9. Figures 3.7A and 3.7B show that the addition of factor on days 5-8 shows a reduced restoration of the antibody responses of both activated and nonactivated lymphocyte cultures. This indicates that factor is required at a relatively early stage in the development of an antibody response to TNP-LPS.

Discussion

The results of this study further characterize the precise cellular requirements of rainbow trout antibody responses by characterizing factors generated upon LPS stimulation of macrophage cell cultures. The accessory activity of an IL-1-like monokine can be measured by the ability of cell-free supernatants to augment the antibody responses *in vitro*. Stimulation with high (200μg/ml) and low (0.2μg/ml) doses of LPS generates supernatants with distinct activities, namely polyclonal activation versus accessory cell function. Stimulation with a low dose of LPS induces the production of an IL-1-like factor which can not polyclonally activate lymphocytes in the absence of antigen, whereas high dose LPS-derived supernatants contain a factor which can polyclonally activate lymphocytes in the presence or absence of antigen. This polyclonal activation is measured by the induction of lymphocyte mitogenesis and antibody production in the absence of antigen. These separate factors can be detected in supernatants as early as 24 hours and are present in optimal levels by day 5 post LPS stimulation of leukocyte cultures. Furthermore, the adsorption of these factors with paraformaldehyde-fixed lymphocytes suggests that both antigen- and non-activated lymphocytes express surface receptors for the polyclonal activating factor(s), whereas only antigen-activated lymphocytes express surface receptors for the low dose LPS-derived IL-1-like factor. Finally, the addition of supernatants to lymphocyte cultures five or more days post stimulation with antigen results in a reduced capacity to stimulate lymphocyte PFC response. These data suggest that there are at least two separate trout cytokines with distinctive activites which can be induced by different doses of LPS.

The initial approach in establishing whether or not rainbow trout macrophages function as accessory cells solely by virtue of secreting a soluble factor (cytokine) was achieved by the use of cell

separation techniques. The ability of the macrophage population of rainbow trout peripheral blood leukocytes to produce an IL-1-like accessory factor was assessed by the ability of cell free supernatants to provide the requisite accessory function for antigen-dependent antibody responses *in vitro*. It was previously reported that TNP-LPS induced PFC responses can be restored by the addition of macrophage-derived cell free supernatants (Ortega and Kaattari, 1993a). These results indicated that at least one requirement of accessory cell function in rainbow trout is the production of a monokine(s), and it was suggested that this monokine is an IL-1 like factor (Ortega and Kaattari, 1993a).

The studies reported here clearly demonstrate the IL-1-like factor dependence of TI B cell responses. In agreement with others, we have found that rigorous macrophage cell depletion methods must be used to demonstrate this fact. In our studies, depletion of adherent macrophages by multiple passages over fibronectin-coated surfaces was found to be an efficient and simple method. In mammalian systems LPS stimulation of macrophages is used for the preparation of IL-1-rich macrophage supernatants (Oppenheim et al., 1986), thus it was logical that LPS may induce IL-1 production in piscine systems as well. However, it has been reported that B cells secrete IL-1 (Scala et al., 1984; Gerrard and Volkman, 1985) or express it in membrane-bound form (Kurt-Jones et al., 1985) under certain conditions, including LPS stimulation. In addition, since it has been shown that fetal calf serum may obscure IL-1 dependence of B cell activation (Hoffman et al., 1984), it is possible that using 2% autologous plasma may have the same effect in our system. Clearly, however, in our experiments any putative activity of lymphocyte-derived IL-1 was not sufficient for inducing or augmenting TI responses, and the use of autologous plasma does not obscure the IL-1-like factor dependence of these PFC responses (Ortega and Kaattari, 1993a).

In this study culture supernatants were generated from rainbow trout PBL stimulated with LPS. One common problem in generating biologically active supernatants is the removal of

mitogen or stimulus source. A technique commonly used to generate mitogen-free supernatants is to briefly pulse cultures with mitogens, but in this technique active factors in supernatants may be removed along with mitogen during the subsequent washing steps. Thus it is necessary to find a window in which the mitogens used can activate cells, but factor production is still at a minimum. Ideally, this will keep factor loss at a minimum while ensuring the removal of mitogen from cultures. In order to remove LPS from culture supernatants while still retaining the IL-1-like factor, the supernatants in this study were filtered using 0.2μm membranes. If a residual amount of LPS was present after filtering, it could not be detected by ^{3}H thymidine incorporation assays (Figure 3.1). Submitogenic or residual doses of LPS have no effect on the TI anti-hapten lymphocyte PFC responses in our assays (Figure 3.1). Thus the active factor(s) in our supernatants are derived from leukocytes and were not introduced exogenously.

In this study low doses of LPS (0.2μg/ml) were used to generate an IL-1-like accessory factor. In contrast, a high dose LPS was capable of generating a polyclonal activating factor. Although supernatants generated from both doses of LPS could restore the PFC responses of lymphocytes cultured in the presence of antigen (TNP-LPS), only high dose LPS-derived supernatants induced the polyclonal activation of lymphocytes in the absence of antigen (Figures 3.2, 3.4). This observed difference may be a result of one or several events. A simple explanation for this is that there is a single or homogenous population of macrophages which are sensitive to different doses of LPS. Upon stimulation with a low dose of LPS, these macrophages produce IL-1, whereas after high dose LPS stimulation or either both IL-1 and polyclonal activating factor or just polyclonal activating factor is produced. Another possibility is based on the assumption that there is a population of low dose LPS-responsive macrophages present in the leukocyte cultures which produce the monokine(s) responsible for inducing the polyclonal activation observed in lymphocyte cultures. Such LPS tolerant macrophages may be unable to respond to low doses of LPS.

Therefore, upon stimulation with low doses of LPS these tolerant macrophages failed to produce the polyclonal activating factor but can produce the IL-1-like factor required for lymphocyte antigen-dependent PFC responses. When stimulated with a highly mitogenic dose of LPS these tolerant macrophages generate the polyclonal activating factor as well as the non-tolerant cells. LPS-specific tolerance has been previously shown to be broken by high doses of LPS (Venkataraman and Scott, 1977) in mammalian studies. This possibility suggests that although the tolerant cells are anergic to stimulus by submitogenic doses of LPS, they are not clonally deleted and can receive stimulatory signals from mitogenic doses of LPS.

Yet another possibilty, although unlikely, is that cellular proliferation is required for the production of the factor(s) which induces polyclonal activation in lymphocyte cultures. In agreement with previously published observations (Kaattari and Yui, 1987), 0.2μg/ml LPS is submitogenic, whereas 200μg/ml LPS is highly mitogenic for trout leukocytes. Although still controversial, it has been reported that B cell proliferation appears to be necessary for the generation of antibody-forming cells in many mammalian *in vitro* model systems (Chace and Scott, 1988; Jelinek and Lipsky, 1983), but this has yet to be reported in piscine systems. However, others have reported that some human polyclonal activators (including LPS) are capable of inducing B cell differentiation into PFCs in the absence of cell division (Grayson et al., 1981).

A more likely explanation of this difference in supernatant activity is that there are at least two populations of responding macrophage subpopulations, one sensitive to low doses LPS and the other requiring high doses of LPS to stimulate factor production. In this study, these distinct subpopulations are producing factors with similar molecular weights but vary in their ability to induce lymphocyte polyclonal activation when cultured without antigen. Moreover, because the of the low yield of macrophages in our culture system, supernatants were generated from unfractionated leukocytes. Thus it is possible that these factors are produced by different cell types. The IL-1-like factor has been previously

identified and shown to be produced by macrophages (Ortega and Kaattari, 1993a). However, it is possible that the polyclonal activating factor present in high dose LPS supernatants is produced by the lymphocyte population in stimulated leukocyte cultures. Rainbow trout lymphocytes have been shown by Graham and Secombes (1990) to secrete a factor which directly effects macrophage function.

The relative activity levels of both the IL-1-like and polyclonal activating factors (Table 3.1) indicate that both supernatants contain similar levels of their respective factors by day 5. The observation that it takes longer for the IL-1-like factor to achieve its optimal level, coupled with the adsorption data in figure 3.4 suggests that there are at least two separate factors produced in these studies. This would indicate that the differences observed between high and low dose supernatants in the receptor adsorption studies (Figure 3.4) are likely due to the presence of separate receptors on non-activated and antigen-activated lymphocytes. This suggests that there is a unique receptor on activated lymphocytes that recognizes the IL-1-like factor, whereas the polyclonal activating factor receptor appears to reside on both activated and non-activated cells. Thus these two factors are distinct. This is not completely unexpected, since carp (*Cyprinus carpio*) leukocytes have been shown to secrete a lymphocyte growth factor which can induce a proliferative response of purified lymphoblasts, but not freshly isolated leukocytes (Caspi and Avtalion, 1984). Furthermore, the activity of this growth factor was reduced following adsorption of the factor-containing supernatants with mitogen-activated blasts (Grondel and Harmsen, 1984).

The incomplete restoration of lymphocyte responses by the polyclonal activating and IL-1-like factors suggest that other factors or mechanisms may be required for inducing optimal PFC responses in our assays. In mammalian systems, several species of accessory cell-derived factors have been reported (reviewed by Corbel and Melchers, 1984). IL-1 itself displays molecular heterogenity and exists in high (35kD), low (17kD), and very low (4kD and 2kD)

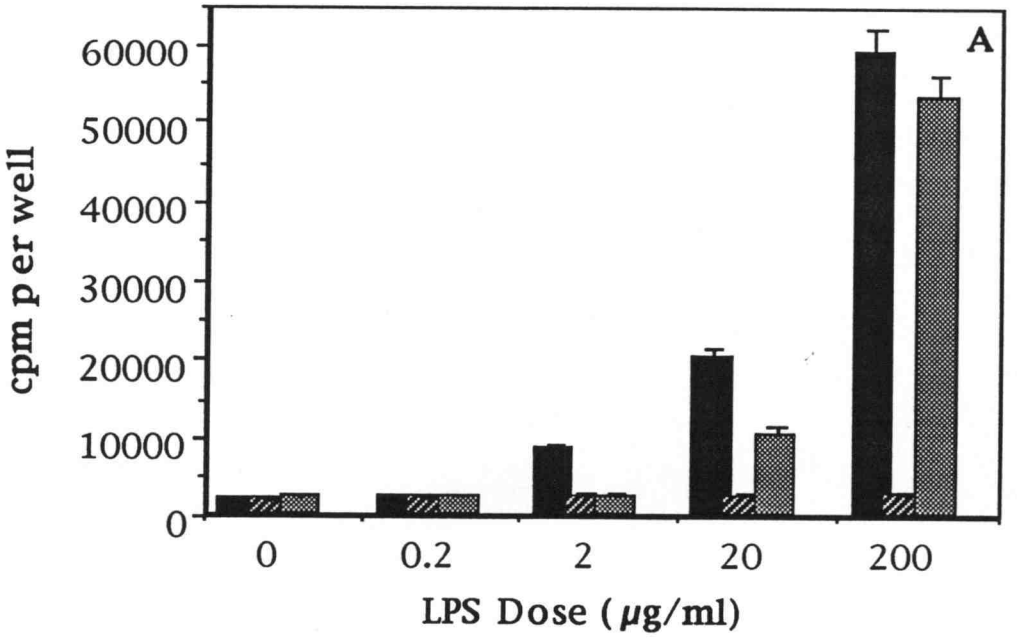
molecular weight forms (Oppenheim et al., 1986). In piscine studies, LPS stimulated channel catfish monocytic cell lines have been shown to produce and secrete high levels of high and low molecular weight species of IL-1 that are active on both catfish and mouse T cells (Vallejo et al., 1991). It is likely that LPS stimulation of rainbow trout leukocytes results in the induction of a cascade of different factors, similar to the acute phase response which has been studied extensively in mammalian systems. The interrelationship of these piscine accessory-cell derived molecules are not clear; whether they display functional heterogeneity remains to be determined.

In piscine lymphocyte TI induced PFC responses IL-1 could function indirectly by helping to initiate a cascade of T cell-derived interleukins, or may function by directly inducing B cells to differentiate into PFCs. Nevertheless, the PFC responses of lymphocytes supplemented with factor-containing supernatants was consistently less than the unfractionated leukocyte controls. There are several possible explanations for this observation. First, some B cells may respond to antigen plus IL-1 alone, whereas others may require both IL-1 and nonspecific T cell factors. Alternatively, IL-1-responsive and T cell factor-responsive B cells may be members of nonoverlapping subsets. Finally, IL-1 may be required for the generation of nonspecific T cell-derived cytokines. Thus the partial restoration of PFC responses by the IL-1-like factor may be due to its action on T cells, more specifically the induction of T cell nonspecific factors. Nevertheless, strong support for a direct role of IL-1 in mammalian B cell activation has been reported by Pike and Nossal (1985) who demonstrated that IL-1 supports the growth and differentiation of a portion of hapten-specific B cells cultured with a TI antigen in the absence of accessory cells or additional cytokines. However, this study has not attempted to address the precise mechanism by which accessory cells may be induced to secrete IL-1.

Acknowledgements

The authors wish to thank Dr. M. Adkison, J. Piganelli, J. Hansen, and P. Wood for their critical review of this manuscript, and J. Bishop and L. Gilkey for technical assistance. This work was supported through the Oregon Sea Grant Award No. R/Aq-60. Agricultural Experiment Station Technical Paper No. 10313.

Figure 3.1: **Removal of LPS in TCM by filtration.** Mitogenic activity of tissue culture medium (TCM) supplemented with increasing LPS doses before and after filtering (0.2μm). Mitogen-induced proliferation (A) and anti-hapten plaque forming cell (PFC; B) leukocyte responses were used to assess the effectiveness of supernatant filtration. Leukocytes were cultured at 10^7 cells/ml in 100μl TCM supplemented with 0.2μg/ml to 200μg/ml LPS. Supernatants were either not filtered (■), filtered immediately after addition of LPS (▨; prefiltered), or filtered after 5 day culture with LPS and leukocytes (▩; post filtered). PFC and mitogen assasys were performed as described in the materials and methods section. The PFCs and cpm were plotted for each medium tested. Each bar represents the mean of three replicate cultures and the error bar represents one standard deviation from the mean.


A
60000
50000
40000
30000
20000
10000
0
cpm p er well
0
0.2
2
20
200
LPS Dose (μg/ml)

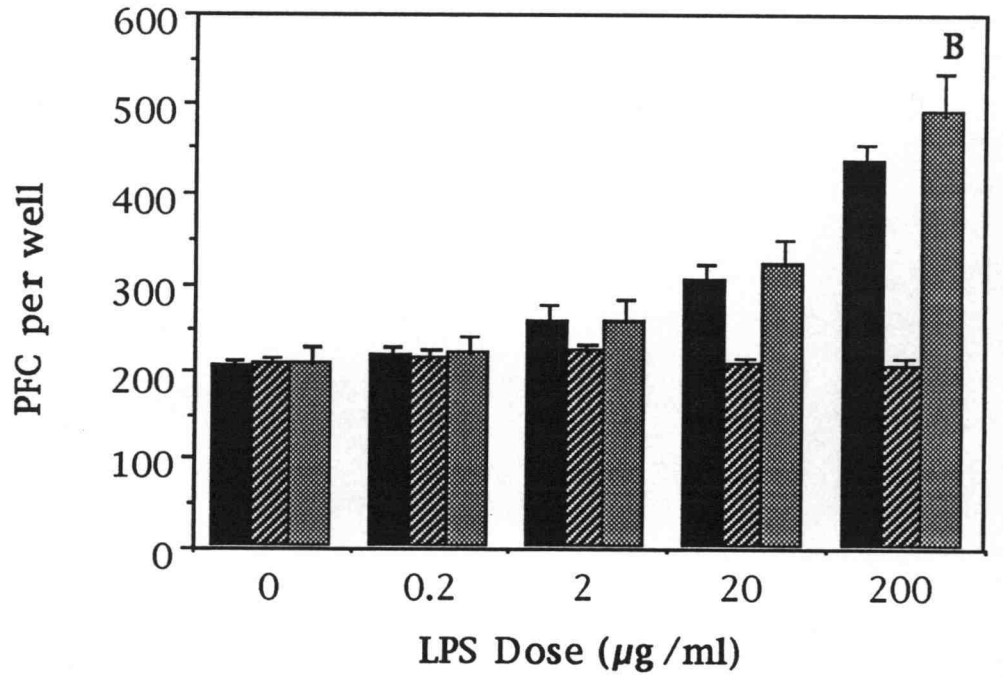

B
600
500
400
300
200
100
0
PFC per well
0
0.2
2
20
200
LPS Dose (μg /ml)

Figure 3.1

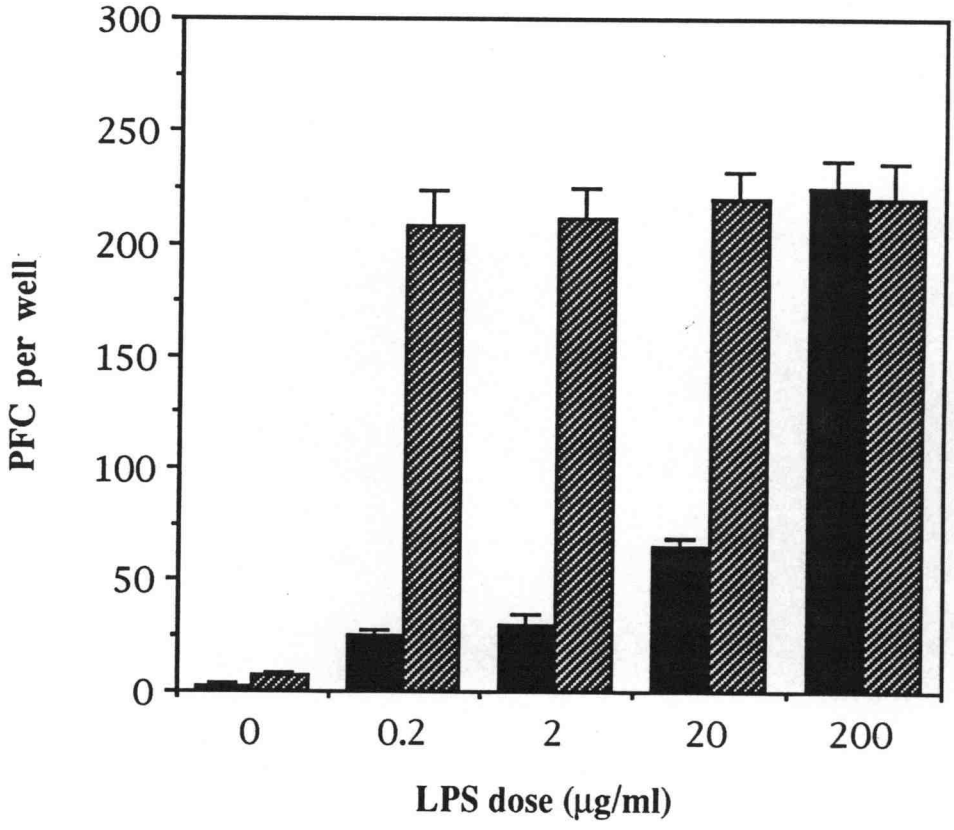


Figure 3.2: **Effect of LPS dose response on factor production.** Supernatants generated from PBL stimulated with 0.2μg/ml to 200μg/ml LPS were assayed for accessory activity in lymphocyte (macrophage-depleted) PFC responses. All supernatants were generated by culturing leukocytes in TCM supplemented with the indicated doses of LPS for 4 days. Upon harvest, all supernatants were filtered with 0.2μm membranes to remove the LPS, and added to lymphocytes upon initiation of cultures. Lymphocytes were cultured at 10^7 cells/ml in TCM supplemented with 50% supernatant (by volume) in the presence (▨) or absence (■) of antigen. The number of PFCs generated per 10^6 cells was plotted for each corresponding culture supernatant tested. Each bar represents the mean of three replicate cultures and the error bar represents one standard deviation from the mean.

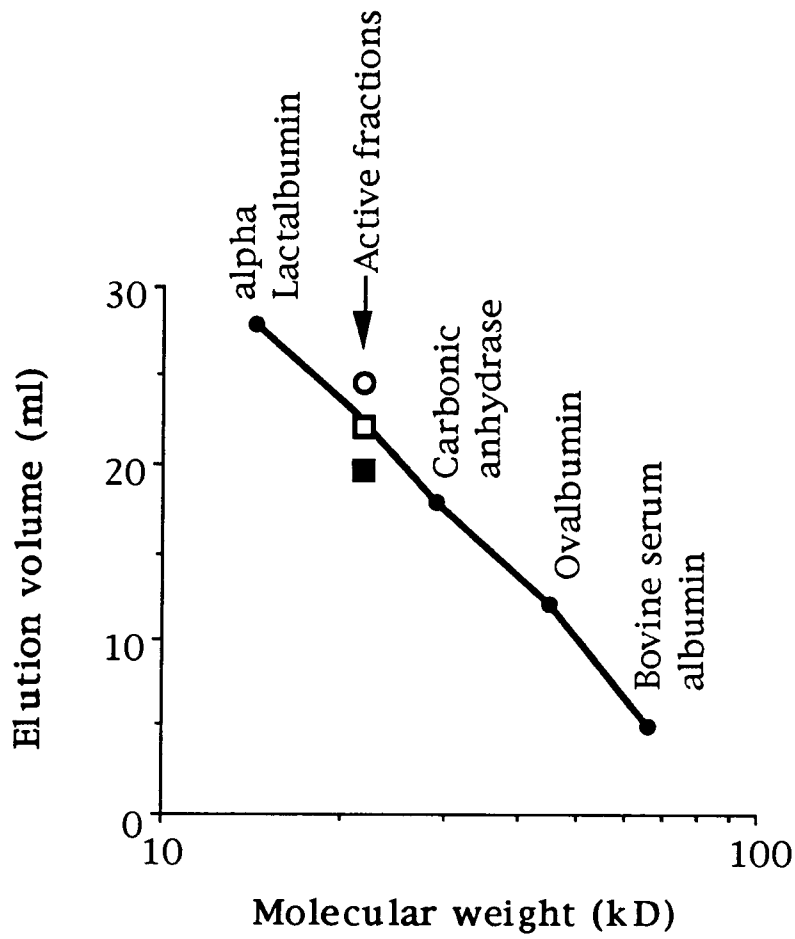


Figure 3.3: **The elution profile for biological activity in fractionated supernatants.** The molecular weight of active fractions in 0.4μg/ml TNP-LPS (O), 0.2μg/ml LPS (□), and 200μg/ml LPS (■) derived supernatants were plotted in addition to the protein standards (●): alpha-lactalbumin (14.2kD), carbonic anhydrase (29kD), ovalbumin (45kD), and bovine serum albumin (66kD).

Figure 3.4: **Adsorption of high (A) and low (B) dose LPS-derived supernatant accessory activity by incubation with paraformaldehyde-fixed lymphocytes.** Supernatants were generated by culturing leukocytes at 10^7 cells/ml in TCM supplemented with a high (200μg/ml) or low (0.2μg/ml) dose of LPS, and harvested on day 5. Supernatants were then adsorbed with 10^8 three day antigen-activated (■) or non-activated (□) and paraformaldehyde-fixed lymphocytes. The supernatants were then evaluated for accessory activity in lymphocyte PFC responses. For comparison, the accessory activity of non-adsorbed supernatants (△) and red blood cell (▲) adsorbed supernatants were also determined. The percent of the maximum PFC response observed (approximately 200 PFC per 10^6 cells) was plotted for each corresponding volume of supernatant used to supplement lymphocyte PFC responses. Each point represents the mean percentage of three replicate cultures.

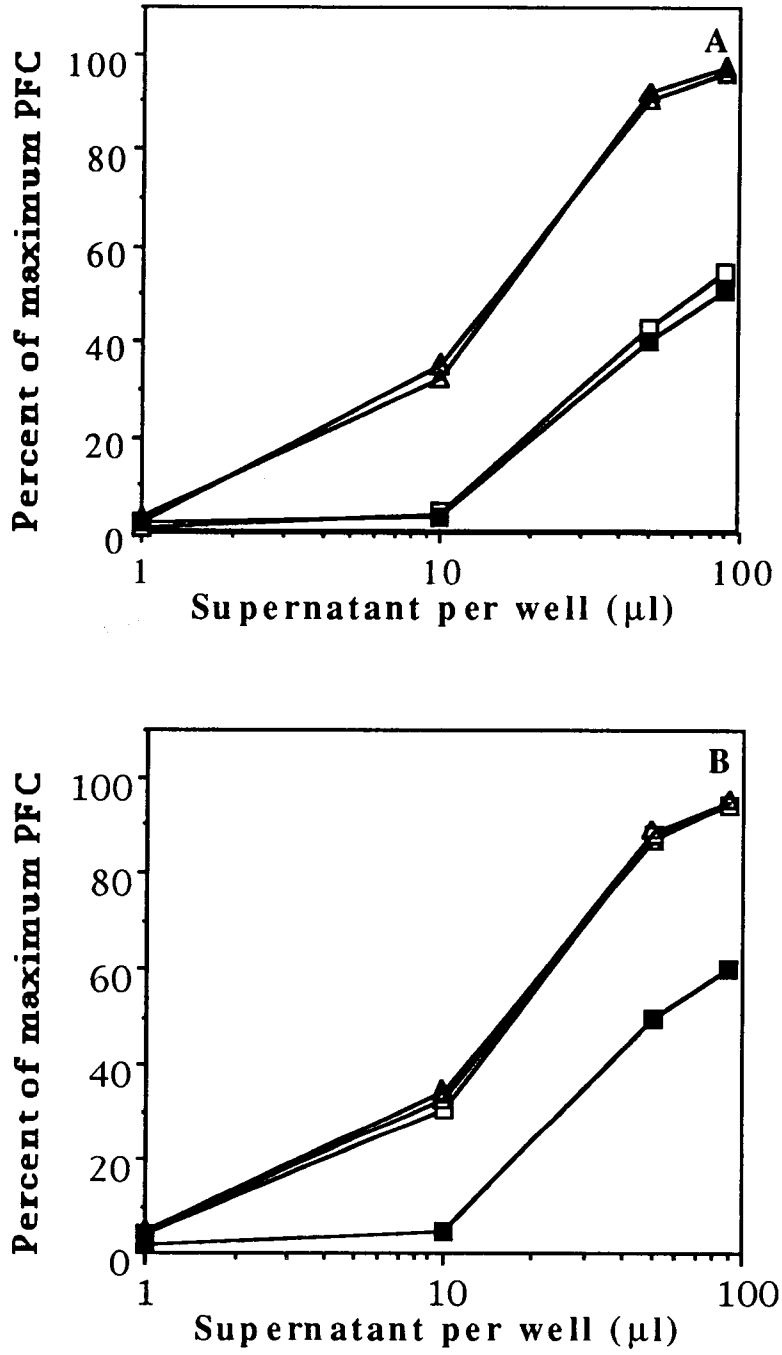

A
Percent of maximum PFC
100
80
60
40
20
0
1
10
100
Supernatant per well (µl)
B
Percent of maximum PFC
100
80
60
40
20
0
1
10
100
Supernatant per well (µl)

Figure 3.4

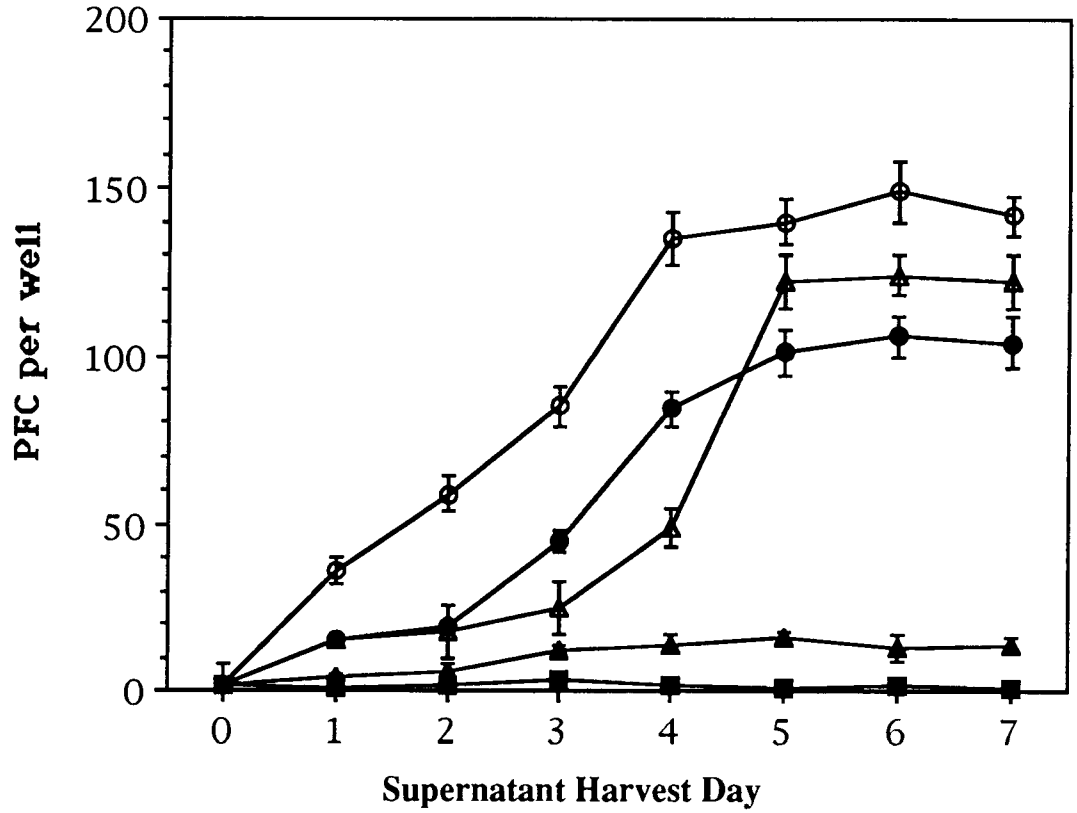


Figure 3.5: **Comparative kinetics of polyclonal activating and IL-1-like factor generation.** Factor containing supernatants were generated by stimulating seven replicate leukocyte cultures containing 10^7 cells in 1 ml TCM with a high (200μg/ml; circles) or low (0.2μg/ml; triangles) dose of LPS. Each supernatant was harvested on the appropriate day, then tested for activity in lymphocyte PFC responses. Lymphocytes were cultured at 10^7 cells/ml in 100μl TCM supplemented with 50% supernatant (by volume) in the presence (open symbols) or absence (filled symbols) of antigen. Control supernatants generated from non-activated leukocytes are represented by filled squares. The number of PFCs generated per 10^6 cells was plotted for each supernatant tested. Each point represents the mean of three replicate cultures and the error bar represents one standard deviation from the mean.

Figure 3.6: **Titration of polyclonal activating (A) and IL-1-like accessory (B) factors.** Factor-containing supernatants were generated by stimulating seven replicate cultures containing 10^7 cells in 1 ml TCM with 200μg/ml or 0.2μg/ml LPS. Supernatants were harvested on day 1 (△), 2 (●), 3 (■), 4 (○), 5 (□), 6 (▲), or 7 (+). Lymphocytes were cultured at 10^7 cells/ml in 100μl TCM supplemented with 50% supernatant (by volume) and 0.4μg/ml TNP-LPS. All cultures were harvested on day 9 and assayed for anti-hapten PFCs. The percent of the maximum PFC response (approximately 200 PFC per 10^6 cells) was plotted for each supernatant tested. Each point represents the mean of three replicate cultures and the error bars represent one standard deviation from the mean.

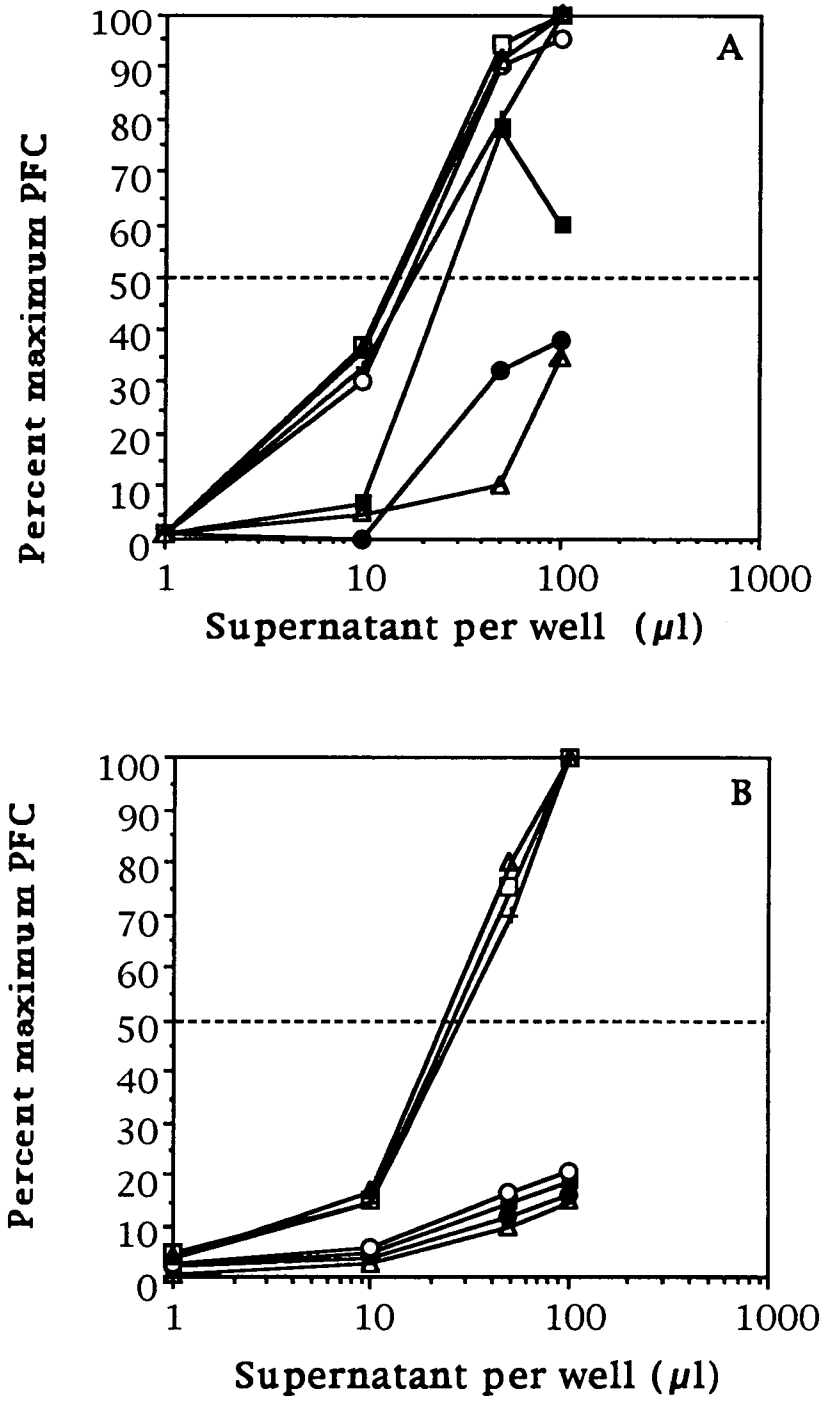

A
B
Percent maximum PFC
100
90
80
70
60
50
40
30
20
10
0
1
10
100
1000
Supernatant per well (μl)

Figure 3.6

	Units of Activity per ml	
Day of Harvest	High (200μg/ml) dose LPS	Low (0.2μg.ml) dose LPS
1	5	1
2	6	1
3	40	5
4	63	5
5	83	50
6	83	63
7	63	50

Table 3.1: **Relative accessory activity of high and low dose LPS-derived supernatants.** The relative accessory activities of supernatants harvested on days 1-7 was determined by titrating the activity of these supernatants. The percent of the maximum PFC response (approximately 200 PFC per 10^6 cells) was plotted for each supernatant tested, and the relative activity of each supernatant was estimated by determining the volume (μl) of supernatant required to give a PFC response equal to 50% of the maximum PFC response observed. This volume (obtained by interpolation of volume of each curve giving 50% of the maximum PFC in figure 3.7) was defined as 1 unit, and all values were converted to units per ml (U/ml).

Figure 3.7: **The addition of high dose (A) and low dose (B) LPS-derived supernatants to lymphocytes on days 0-8 of the PFC response.** Lymphocytes were cultured at 10^7 cells/ml in 100μl TCM supplemented with (▨) or without (■) antigen. High or low dose supernatant (100μl) was added to appropriate cultures on days 0-8, and all cultures were harvested and assayed for PFCs on day 9. The number of PFCs generated per 10^6 cells was plotted for each corresponding day of supernatant addition. Each bar represents the mean of three replicate cultures and the error bar represents one standard deviation from the mean.

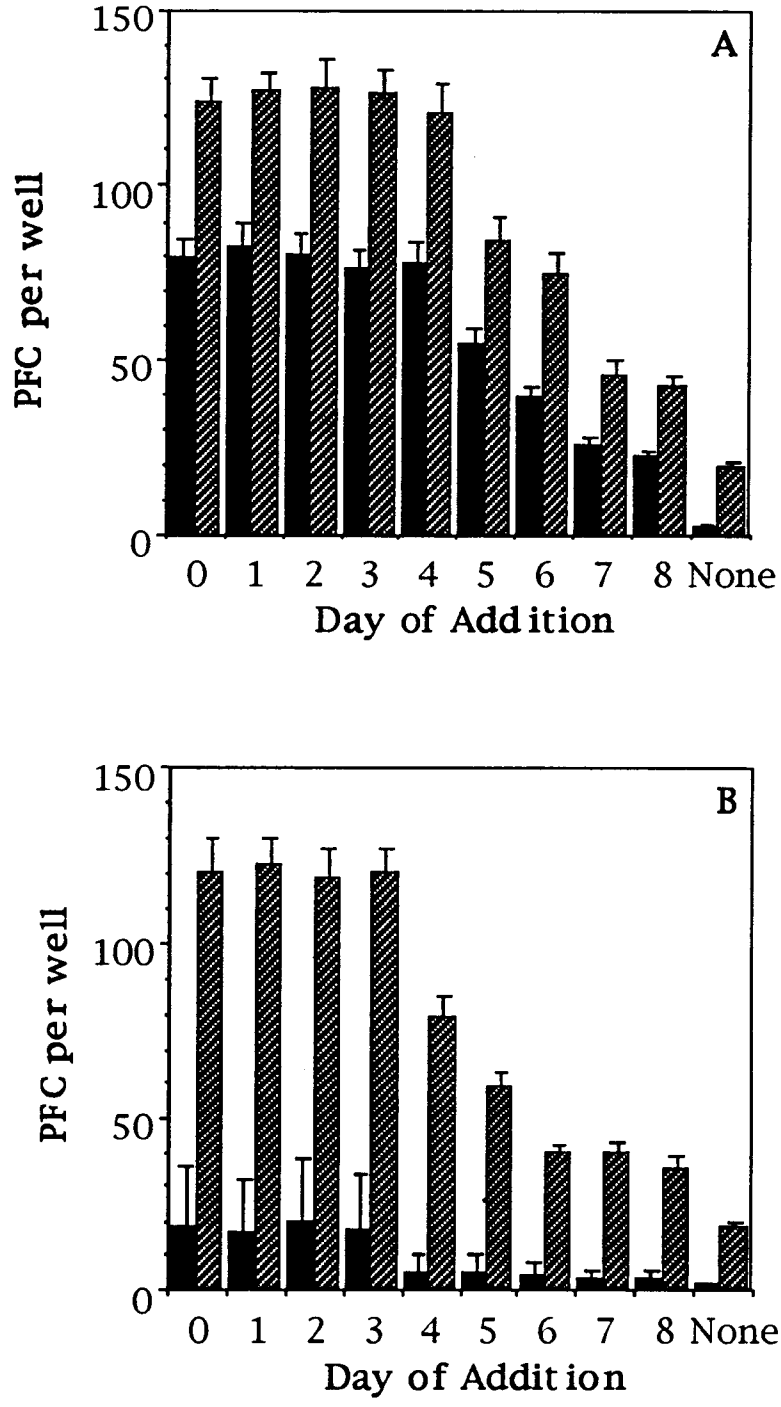

150
100
50
0
PFC per well
A
0 1 2 3 4 5 6 7 8 None
Day of Addition
150
100
50
0
PFC per well
B
0 1 2 3 4 5 6 7 8 None
Day of Addition

Figure 3.7

CHAPTER 4

Conclusions

Elucidation of the interactions between piscine leukocytes in response to antigen has relied heavily upon defined hapten-carrier systems, *in vitro* leukocyte culture systems, and cell partitioning techniques, all of which were originally developed for mammalian systems. Recent advances in piscine leukocyte culture systems permit the use of limiting dilution analysis (LDA) as an alternative method for assessing cellular cooperation between leukocytes upon induction by antigen. This method, unlike the more conventional methods used, allows for the elucidation of the sequence of B cell maturation and antigen-induced differentiation associated with B cell responses at both early and late phases.

In this study the ability of different subpopulations of rainbow trout (*Oncorynchus mykiss*) peripheral blood leukocytes to respond to the T-independent antigen trinitrophenylated-lipopolysaccharide (TNP-LPS) was assessed by using an *in vitro* passive hemolytic plaque assay. In chapter 2 it was demonstrated that macrophages are required for providing an accessory function during the antibody response to this antigen. Establishment of this accessory function was demonstrated by showing that macrophages (adherent leukocytes) were able to restore the capacity for antibody production to purified lymphocytes (non-adherent leukocytes). Furthermore, supernatants from antigen-stimulated macrophages were sufficient to restore lymphocyte function. The use of limiting dilution analysis confirmed this requisite role of the macrophage-derived factor, revealed that the target of this factor(s) is the B cell

precursor, and suggests that there may be differential sensitivity of B cell precursors to this factor. Finally, the use of non-haptenated LPS verified that LPS alone is the required stimulus for inducing this factor. Based on the factor source, function, and molecular weight it was suggested that IL-1 is the requisite accessory factor.

In chapter 3 a novel polyclonal activating factor was identified by stimulating leukocytes with high doses of LPS. The activity of this factor and the IL-1-like factor activity, as measured by the provision of accessory function in lymphocyte PFC responses, is produced as early as 24 hours and at optimal levels by day 5 post LPS stimulation of leukocyte cultures. Stimulation with different doses of LPS-generated supernatants with different activities, as observed by polyclonal activation of lymphocytes. Stimulation of leukocytes with a low dose (0.2μg/ml) of LPS induced the production of the IL-1-like factor, whereas supernatants generated with a high dose (200μg/ml) of LPS produces a factor which induced polyclonal activation of lymphocytes. This difference in activity was also demonstrated by the adsorption of supernatants onto paraformaldehyde-fixed lymphocytes. High dose LPS supernatant activity was reduced following adsorption with either non-activated or antigen-activated lymphocytes, whereas low dose LPS supernatant accessory activity was reduced only when adsorbed with antigen-activated lymphocytes. This suggests that lymphocyte surface receptors for the polyclonal activating factor are present on both antigen- and non-activated lymphocytes, while only activated lymphocytes express a surface receptor for the IL-1-like accessory factor. Finally, the addition of factors to cultures at later stages of the PFC response decreased its activity in lymphocyte PFC responses. The similarities of this trout IL-1-like factor and mammalian IL-1 have been discussed, and it has been proposed that this is the first demonstration of a salmonid monokine analogous to mammalian IL-1 as well as a newly identified cytokine which both affect B cell function.

BIBLIOGRAPHY

Al-Adhami, M.A., and Kunz, Y.W. 1976. Haemopoietic centres in the developing angelfish *Pterophyllum scalare* (Curier and Valenciennes). Wilheim Roux's Arch. 179: 393-401.

Allen, J.N., Herzyk, D.J., Allen, E.D., and Wewers, M.D. 1992. Human whole blood interleukin-1 beta production: kinetics, cell source, and comparison with TNF-alpha. J. Lab. Clin. Med. 119: 538-546.

Anderson, D.P. 1974. Fish Immunology. TFH Publications: Neptune.

Andersson, J., Bjork, L., Dinarello, C.A., Towbin, H., and Andersson, U. 1992. Lipopolysaccharide induces human IL-1 receptor antagonist and IL-1 production in the same cell. Eur. J. Immunol. 22: 2617-2623.

Andrew, T.A. and Owen, J.J. 1978. Studies on the earliest sites of B cell differentiation in the mouse embryo. Dev. Comp. Immunol. 2: 339-346.

Arend, W.P. 1991. Interleukin-1 receptor antagonist. A new member of the interleukin-1 family. J. Clin. Invest. 88: 1455-1451.

Arkoosh, M. 1990. Development of immunological memory in rainbow trout *(Oncorhynchus mykiss)* and aflatoxin B_1 modulation of the response. Doctoral Thesis, Oregon State University. Corvallis, Oregon.

Arkoosh, M , and Kaattari S.L. 1991. Development of immunological memory in rainbow trout (*Oncorhynchus mykiss*). 1. An immunochemical and cellular analysis of the B cell response. Dev. Comp. Immunol. 15: 279-293.

Beuscher, H.U., and Colten, H.R. 1988. Structure and function of membrane IL-1. Mol. Immunol. 25: 1189-1199.

Beuscher, H.U., Nickells, M.W., and Colten, H.R. 1988. The precursor of interleukin-1 alpha is phosphorylated at residue serine 90. J. Biol. Chem. 263: 4023-4028.

Beutler, B., and Cerami, A. 1988. The history, properties, and biological effects of cachectin/TNF alpha, primary mediator of the host response. Biochemistry 27: 7575-7582.

Blazer, V.S., Wolke, R.E., Brown, J., and Powell, C.A. 1987. Piscine macrophage aggregate parameters as health monitors: Effect of age, sex, relative weight, season, and site quality in largemouth bass (*Micropterus salmoides*). Aquatic Toxicol. 10: 299-215.

Bly, J.E., and Clem, L.W. 1991. Temperature-mediated processes in teleost immunity: *In vitro* immunosuppression induced by *in vivo* low temperature in channel catfish. Vet. Immunol. Immunopath. 28: 365-377.

Bomalski, J.S., Steiner, M.R., Simon, P.L., and Clark, M.A. 1992. IL-1 increases phospholipase A2 activity, expression of phospholipaseA2-activating protein, and release of linoleic acid from the murine T helper cell line EL-4. J. Immunol. 148: 155-160.

Bomsztyk, K., Sims, J.E., Stanton, T.H., Slack, J., McMahan, C.J., Valentine, M.A., and Dower, S.K. 1989. Evidence for different interleukin-1 receptors in murine B- and T-cell lines. Proc. Nat. Acad. Sci. 86: 8034-8039.

Bonnefoy, J.Y., Denoroy, M.C., Guillot, O., Martens, C.L., and Banchereau, J. 1989. Activation of normal human B cells through their antigen receptor induces membrane expression of IL-1 alpha and secretion of IL-1 beta. J. Immunol. 143: 864-869.

Braciale, T.J., Morrison, L.A., Sweetser, M.T., Sambrook, J., Gething, M., and Braciale, V.L. 1987. Antigen presentation pathways to class I and class II MHC-restricted T lymphocytes. Immunol. Rev. 98: 97-114.

Brevario, F., d'Aniello, E.M., Golay, J., Peri, G., Bottazzi, B., and Rocchi, M. 1992. Interleukin-1 inducible genes in endothelial cells. Cloning of a new gene related to C-reactive protein and serum amyloid P component. J. Biol. Chem. 267: 22190-22197.

Burke, R.D., and Watkins, R.F. 1991. Stimulation of starfish coelomocytes by interleukin-1. Biochem. Biophys. Res. Commun. 180: 579-584.

Cameron, P., Limjuco, G., Rodkey, J., Bennett, C., and Schmidt, J.A. 1985. Amino acid sequence and analysis of human interleukin-1 (IL-1). Evidence for biochemically distinct forms of IL-1. J. Exp. Med. 162: 790-801.

Caspi, R.R., and Avtalion, R.R. 1984. Evidence for existence of an IL-2-like lymphocyte growth promoting factor in the bony fish, *Cyprinus carpio.* Dev. Comp. Immunol. 8: 51-60.

Chace, J.H., and Scott, D.W. 1988. Activation events in hapten-specific B cells from tolerant mice. J. Immunol. 141: 3258-3262.

Chain, B.M., Kay, P.M., and Shaw, M. 1988. The biochemistry and cell biology of antigen processing. Immunol. Rev. 106: 33-58.

Chen, C., Bruderer, U., and Rittenberg, M.B. 1992. The developmental patterns of B cell precursors distinguishing between environmental and nonenvironmental forms of phosphocholine. Cell. Immunol. 143: 378-388.

Chesnut, R.W., and Grey, H.W. 1985. Antigen presenting cells and mechanisms of antigen presentation. CRC Crit. Rev. Immunol. 5: 263-316.

Chiller, J.M., Habicht, G.S., and Weigle, W.O. 1971. Kinetic differences in unresponsiveness of thymus and bone marrow cells. Science 171: 813-817.

Chiller, J.M., Hodgins, H.O., Chambers, V.C. and Weiser, R.S. 1969a. Antibody response in rainbow trout (*Salmo gairdneri*). I. Immunocompetent cells in the spleen and anterior kidney. J. Immunol. 102: 1193-1201.

Chiller, J.M., Hodgins, H.O., and Weiser, R.S. 1969b. Antibody response in rainbow trout (*Salmo gairdneri*). II. Studies on the kinetics of development of antibody-producing cells and on complement and natural hemolysin. J. Immunol. 102: 1202-1207.

Chilmonczyk, S. 1992. The thymus in fish: Development and possible function in the immune response. Ann. Rev. Fish Dis. 2: 181-200.

Chilmonczyk, S. 1983. The thymus of the rainbow trout *Salmo gairdneri.* Light and electron microscopic study. Dev. Comp. Immunol. 7: 59-68.

Chizzonite, R., Truitt, T., Nunes, P., Lugg, D.K., and Gubler, U. 1989. Two high affinity interleukin-1 receptors represent separate gene products. Proc. Nat. Acad. Sci. 86: 8029-8033.

Clem, L.W., Miller, N.W., and Bly, J.E. 1991. Evolution of lymphocyte subpopulations, their interactions and temperature sensitivities. In: Warr, G., Cohen, N. (editors). Phylogenesis of immune functions. CRC Press, Boca Raton, FL. Pages 191-214.

Clem, L.W., Sizemore, R.C., Ellsaesser, C.F., and Miller, N.W. 1985. Monocytes as accessory cells in fish immune responses. Dev. Comp. Immunol. 9: 803-809.

Conti, P., Panara, M.R., Porrini, A.M., Barbacane, R.C., Bongrazio, M., and Dempsey, R.A. 1992a. Inhibition of interleukin-1 (alpha and beta), interleukin-2 secretion, and surface expression of interleukin-2 recpetor by a novel cytokine interleukin-1 receptor antagonist (IL-1ra). Scand. J. Immunol. 36: 27-33.

Conti, P., Barbacane, R.C., Panara, M.R., Reale, M., Placido, F.C., Fridas, S., Bongrazio, M., and Dempsey, R.A. 1992b. Human recombinant interleukin-1 receptor antagonist (hrIL-1ra) enhances the stimulatory effect of IL-2 on natural killer cell activity against MOLT-4 target cells. Int. J. Immunopharmacol. 14: 987-993.

Conti, P., Feliciani, C., Barbacane, R.C., Panara, M.R., Reale, M., Placido, F.C., Sauder, D.N., Dempsey, R.A., and Amerio, P. 1992c. Inhibition of interleukin-1 beta mRNA expression and interleukin-1 alpha and beta secretion by a specific human recombinant interleukin-1 receptor antagonist in human peripheral blood mononuclear cells. Immunology 77: 245-250.

Corbel, C., and Melchers, F. 1984. The synergism of accessory cells and of soluble alpha-factors derived from them in the activation of B cells to proliferation. Immunol. Rev. 78: 51-72.

Cuchens, M.A., and Clem, L.W. 1977. Phylogeny of lymphocyte heterogeneity II. Differential effects of temperature of fish T-like and B-like cells. Cell. Immunol. 34: 219-230.

Dayton, E.T., Matsumoto-Kobayashi, M., Perussia, B., and Trinchieri, G. 1985. Role of immune interferon in the monocytic differentiation of human promyelocytic cell lines induced by leukocyte conditioned medium. Blood 66: 583-594.

de Kinkelin, P., Dorson, M., and Hatenberger-Baudouy, A.M. 1982. Interferon synthesis in trout and carp after viral infection. Dev. Comp. Immunol. 2: 167-174.

DeGrado, W.F., Wasserman, Z.R., and Chowdhry, V. 1991. Sequence and structural homologies among type I and type II interferons. Nature 300: 379-381.

DeKoning, J., and Kaattari, S.L. 1991. Mitogenesis of rainbow trout peripheral blood lymphocytes requires homologous plasma for optimal responsiveness. In Vitro 27A: 381-386.

DeLuca, D., Wilson, M., and Warr, G.W. 1983. Lymphocyte heterogeneity in the trout, *Salmo gairdneri*, defined with monoclonal antibodies to IgM. Eur. J. Immunol. 7: 546-551.

Derynck, R., Jarret, J.A., Chen, E.Y., Eaton, D.H., Bell, J.R., Assoian, R.K., Roberts, A.B., Sporn, M.B., and Goddel, D.V. 1985. Human transforming growth factor beta: Complementary DNA sequence and expression in normal and transformed cells. Nature 316: 701-705.

Desena, J., and Rio, G.J. 1975. Partial purification and characterization of RTG-2 fish cell interferon. Infect. Immunol. 11: 815-818.

di Giovine, F., and Duff, G.W. 1990. Interleukin 1: The first interleukin. Immunol. Today 11: 13-20.

Dinarello, C.A. 1990. Interleukin-1 and its biologically related cytokines. In: Cohen, S. (editor). Lymphokines and the immune response. CRC Press, Boca Raton, FL. Pages 145-180.

Dinarello, C.A. 1985. An update on human interleukin-1: From molecular biology to clinical relevance. J. Clin. Immunol. 5: 287-297.

Dumonde. D.C., Wolstencroft, R.A., Panayi, G.S., Mathew, M., Morely, J., and Howson, W.T. 1969. Lymphokines: Non-antibody mediators of cellular immunity generated by lymphocyte activation. Nature 38: 2245-2249.

Dutton, R.W., and Swain, S.L. 1987. B cell and T cell growth factors. In: Boynton, A.L., and H.L. Leffert (editors) Control of animal cell proliferation. Academic Press, Inc. New York, NY. Pages 219-261.

Ellis, A.E. 1980. Antigen-trapping in the spleen and kidney of the plaice, *Pleuronectes platessa L.* J. Fish Dis. 3: 413-416.

Ellis, A.E. 1977. Ontogeny of the immune response in *Salmo salar.* Histogenesis of the lymphoid organs and appearance of membrane immunoglobulin and mixed leucocyte reactivity. In: Solomon, J.B., and Horton, J.D. (editors) Developmental Immunobiology. Elsevier/North-Holland Biomedical Press, Amsterdam. Pages 225-231.

Ellis, A.E., and de Sousa, M. 1974. Phylogeny of the lymphoid system. I. A study of the fate of circulating lymphocytes in plaice. Eur. J. Immunol. 4: 338-343.

Ellis, A.E., Munroe, A.L.S., and Roberts, R.J. 1976. Defence mechanisms in fish I. A study of the phagocytic system and the fate of intraperitoneally injected particulate material in the plaice (*Pleuronectes platessa*). J. Fish Biol. 8: 67-78.

Ellsaesser, C.F.M. 1989. Identification and characterization of high and low molecular weight species of channel catfish IL-1 with differential activities on fish and mouse lymphocytes. Doctoral dissertation, University of Mississippi. Jackson, Mississippi.

Ellsaesser, C.F., Bly, J.E., and Clem, L.W. 1988. Phylogeny of lymphocyte heterogeneity: The thymus of the channel catfish. Dev. Comp. Immunol. 12: 787-799.

Fibbe, W.E., Van-Damme, J., Billiau, A., Duinkerken, N., Lurvink, E., Ralph, P., Altrock, B.W., Kaushansky, K., Willemze, R., and Falkenburg, J.H. 1988. Human fibroblasts produce G-CSF, M-CSF, and GM-CSF following stimulation by interleukin-1 and poly(rI) poly(rC). Blood 72: 860-866.

Freitas, A.A., Pereira, P., Huetz, F., Thomas-Vaslin, V., Pena-Rossi, C., Andrade, L., Sundblad, A., Forni, L., and Coutinho, A. 1989. B cell activities in normal unmanipulated mice. In: del Guercio, P. and Cruse, J.M. (editors) B Lymphocytes: Function and Regulation. Pages 1-26.

Freitas, A.A., Rocha, B., Forni, L., and Coutinho, A. 1982. Population dynamics of B lymphocytes and their precursors: demonstration of high turnover in the central and peripheral lymphoid organs. J. Immunol. 128: 54-60.

Friedman, R.M., and Vogel, S.N. 1983. Interferons with special emphasis on the immune system. Adv. Immunol. 34: 97-140.

Furutani, Y., Notake, M., Yamayoshi, M., Yamagishi, J., Nomura, H., Ohue, M., Furtura, R., Fukui, T., Yamada, M., and Nakamura, S. 1985. Cloning and characterization of the cDNAs for human and rabbit interleukin-1 precursor. Nucl. Acids Res. 16: 5869-5882.

Gathings, W.E., Lawton, A.R., and Cooper, M.D. 1977. Immunoflourescent studies of the development of pre-B cells, B lymphocytes and immunoglobulin isotype diversity in humans. Eur. J. Immunol. 7: 804-810.

Gerrard, T.L., and Volkman, D.J. 1985. IL-1-like activity in antigen-presenting human B cell lines. Immunology. 12: 197-202.

Grace, M.F. and Manning, M.J. 1980. Histogenesis of the lymphoid organs in rainbow trout *Salmo gairdneri.* Dev. Comp. Immunol. 4: 255-264.

Graham, S., and Secombes, C.J. 1990a. Cellular requirements for lymphokine secretion by rainbow trout *Salmo gairneri* leukocytes. Dev. Comp. Immunol. 14: 59-68.

Graham, S., and Secombes, C.J. 1990b. Do fish lymphocytes secrete interferon? J. Fish Biol. 36: 563-573.

Graham, S., and Secombes, C.J. 1990c. Cellular requirements for lymphokine secretion by rainbow trout , *Salmo gairdneri* leukocytes. Dev. Comp. Immunol. 14: 59-68.

Graham, S., and Secombes, C.J. 1988. The production of a macrophage-activating factor from rainbow trout *Salmo gaidneri* leukocytes. Immunology 65: 293-297.

Gray, P.W., Glasiter, D., Chen, E., Goeddel, D.V., and Pennica, D. 1986. Two interleukin-1 genes in the mouse: Cloning and expression of the cDNA for murine interleukin-1 beta. J. Immunol. 137: 3644-3648.

Gray, P.W., and Goeddel, D.L. 1983. Cloning and expression of murine immune interferon cDNA. Proc. Nat. Acad. Sci. 80: 5842-5846.

Gray, P.W., and Goeddel, D.L. 1982. Structure of the human immune interferon gene. Nature 298: 859-863.

Grayson, J., Dooley, N.J., Koski, I.R., and Blaese, R.M. 1981. Immunoglobulin production induced in vitro by glucocorticoid hormones. T cell-dependent stimulation of immunoglobulin production without B cell proliferation in cultures of human peripheral blood lymphocytes. J. Clin. Invest. 68: 1539-1543.

Grondel, J.L., and Harmsen, E.G.M. 1984. Phylogeneity of interleukins: Growth factors produced by leucocytes of the cyprinid fish, *Cyprinus carpio* L. Immunology 52: 477-482.

Gubler, U., Chua, A.O., Stern, A.S., Hellman, C.P., Vitek, M.P., DeChiara, T.M., Benjamin, W.R., Collier, K.J., and Dukovich, M. 1986. Recombinant human interleukin-1 alpha: Purification and biological characterization. J. Immunol. 136: 2492-2497.

Hagicuara, H., Huang, H.J., Arai, N., and Zlotmka, A. 1987. IL-1 modulates messenger RNA levels of lymphokines and other molecules associated with T cell activation in the T cell lymphoma LBRM33-IA5. J. Immunol. 138: 2514-2519.

Hamby, B.A., Huggins, Jr., E.M., Lachman, L.B., Dinarello, C.A., and Sigel, M.M. 1986. Fish lymphocytes respond to human IL-1. Lymphokine Res. 5: 157-162.

Hannum, C.H., Wilcox, C.J., Arend, W.P., Joslin, F.G., Dripps, D.J., Heimdal, P.L., Armes, L.G., Sommer, A., Eisenberg, S.P., and Thompson, R.C. 1990. Interleukin-1 receptor antagonist activity of a human interleukin-1 inhibitor. Nature 343: 336-340.

Harada, N., Matsumoto, M., Koyama, N., Scimizu, A., Honjo, T., Tominaga, A., and Takatsu, K. 1987. T cell replacing factor/interleukin 5 induces not only B cell growth and differentiation, but also increased expression of IL-2 receptor on activated B cells. Immunol. Lett. 15: 205-215.

Harding, C.V. 1991. Pathways of antigen processing. Curr. Opinion Immunol. 3: 3-9.

Harding, C.V., Leyva-Cobian, F., and Unanue, E.R. 1988. Mechanisms of antigen processing. Immunol. Rev. 106: 77-92.

Harding, C.V., Unanue, E.R., Slot, J.W., Schwartz, A.L., and Geuze, H.J. 1990. Functional and ultrastructural evidence for intracellular formation of major histocompatibility complex Class II-peptide complexes during antigen processing. Proc. Nat. Acad. Sci. 87: 5553-5557.

Hayward, A.R., Simonse, M.A., Lawton, A.R., Mage, R.G. and Cooper, M.D. 1978. Pre-B and B cells in rabbits: ontogeny and allelic exclusion of kappa light chain genes. J. Exp. Med. 148: 1367-1377.

Hazuda, D.J., Strickler, J., Simon, P., and Young, P.R. 1991. Structure-function mapping of interleukin-1 precursors. Cleavage leads to a conformational change in mature protein. J. Biol. Chem. 266: 7080-7086.

Henco, K., Brosius, J., Fujisawa, A., Fujisawa, J.-I., Haynes, J.R., and Weissmann, C. 1985. Structural relationships of human interferon alpha genes and pseudogenes. J. Molec. Biol. 185: 227-260.

Herbelin, A., Schneider, E., Papiernik, M., and Dy, M. 1992. IL-7 is requisite for IL-1-induced thymocyte prloiferation. Involvement of IL-7 in the synergistic effects of GM-CSF or TNF with IL-1. J. Immunol. 148: 99-105.

Hestdal, K., Jacobsen, S.E., Dubois, C.M., Chizzonite, R., Oppenheim, J.J., and Keller, J.R. 1992. *In vivo* effect of interleukin-1 alpha on hematopoiesis: Role of colony-stimulating factor receptor modulation. Blood. 80: 2486-2494.

Hoffman, M.K., Mizel, S.B., Hirst, J.A. 1984. IL-1 requirement for B cell activation revealed by the use of adult serum. J. Immunol. 133: 2566-2570.

Holbrook, N.J., Smith, K.A., Fornace, A.J.J., Comeau, C.M., Wiskoch, R.L., and Crabtree, G.R. 1984. T cell growth factor: Complete nucleotide sequence and organization of the gene in normal and malignant cells. Proc. Nat. Acad. Sci. 81: 1634-1638.

Holter, W., Goodman, C.K., Casabo, L., Nelson, D.L., Greene, W.C., and Waldmann, T.S. 1987. Expression of functional IL-2 receptors by lipopolysaccharide and interferon gamma-stimulated human monocytes. J. Immunol. 138: 2917-2992.

Hughes, T.K., Smith, E.M., Barnett, J.A., Charles, R., Stefano, G.B. 1991. LPS stimulated invertebrate hemocytes: A role for immunoreactive TNF and IL-1. Dev. Comp. Immunol. 15: 117-122.

Ihrie, E.J., and Wood, D.D. 1985. Biochemical heterogeneity of human interleukin-1. Lymphokine Res. 4: 169-181.

Inaba, K., Kitaura, M., Kato, T., Watassabe, Y., Kawade, Y., and Muramatsu, S. 1986. Contrasting effects of alpha, beta, and gamma interferons on the expression of macrophage Ia antigens. J. Exp. Med. 163: 1030-1035.

Inaba, K., and Steinman, R.M. 1986. Accessory cell-T lymphocyte interactions. Antigen-dependent and -independent clustering. J. Exp. Med. 163: 247-261.

Irwin, M.J. and Kaattari, S.L. 1986. Salmonid B lymphocytes demonstrate organ dependent functional heterogeneity. Vet. Immunol. and Immunopath. 12: 39-45.

Isakson, P., Pure, E., Vitteta, E., and Krammer, P. 1982. T cell derived B cell differentiation factor(s). Effect on the isotype switch of murine B cells. J. Exp. Med. 155: 734-741.

Iwasaki, T., Graves, L., Rachie, N., and Bomsztyk, K. 1992. Herbimycin A blocks IL-1-induced NF-kappa B DNA binding activity in lymphoid cell lines. FEBS lett. 298: 240-244.

Jacobs, D.M., and Morrison, D.C. 1975. Stimulation of T-independent primary anti-hapten response *in vitro* by TNP-LPS. J. Immunol. 144: 360-369.

Jelinek, D.F., and Lipsky, P.E. 1987. Regulation of human B lymphocyte activation, proliferation, and differentiation. Adv. Immunol. 40: 1-59.

Jelinek, D.F., and Lipsky, P.E. 1983. The role of B cell proliferation in the generation of immunoglobulin-secreting cells in man. J. Immunol. 130: 2597-2604.

Jensen, P.E. 1990. Regulation of antigen presentation by acidic pH. J. Exp. Med. 171: 1779-1784.

Kaattari, S.L. 1992. Fish B lymphocytes: Defining their form and function. Ann. Rev. Fish Dis. 2: 161-180.

Kaattari, S.L., and Irwin, M.J. 1985. Salmonid spleen and anterior kidney harbor populations of lymphocytes with different B cell repertoires. Dev. Comp. Immunol. 9: 433-444.

Kaattari, S.L., and Yui, M.A. 1987. Polyclonal activation of salmonid B lymphocytes. Dev. Comp. Immunol. 11: 155-165.

Kasahara, T., Hooks, J.J., Dougherty, S.F., and Oppenheim, J.J. 1983. Interleukin-2-mediated immune interferon (IFN-gamma) production by human T cells and T cell subsets. J. Immunol. 130: 1784-1789.

Kelso, A., Gough, N.M., and Metcalf, D. 1990. Production of hemopoietic colony stimulating factors by murine T lymphocytes. In: Cohen, S. (editor). Lymphokines and the immune response. CRC Press, Boca Raton, FL. Pages 213-236.

Kessler, D.J., Duyao, M.P., Spicer, D.B., and Sonenshein, G.E. 1992. NF-kappa B-like factors mediate interleukin-1 induction of c-myc gene transcription in fibroblasts. J. Exp. Med. 176: 787-792.

Kinashi, T., Harada, N., Severinson, E., Tanabe, T., Sideras, P., Konishi, M., Azuma, C., Tminaga, A., Bergatedt-Lindqvist, S., Takahashi, M., Natsuda, F., Yaotia, Y., Takatsu, K., and Honjo, T. 1986. Cloning of complementary DNA encoding T cell replacing factor and identity with B cell growth factor II. Nature 324: 70-73.

Kishimoto, T., Taga, T., Matsuda, T., Hibi, M., Suematsu, S., Tang, B., Yawata, H., Hirata, Y., Yamaski, K., and Hirano, T. 1990. Interleukin-6 and its receptor in immune regulation. Prog. Immunol. 7: 633-654.

Kroese, F.G.M. 1983. Antigen trapping in the spleen of the red-eared turtle. Dev. Comp. Immunol. 7: 711-712.

Kroese, F.G.M., Leceta, J., Dopp, E.A., Herraez, M.P., Nieuwenhuis, P., and Zapata, A. 1985. Dendritic immune complex trapping cells in the spleen of the snake, *Python reticulatus.* Dev. Comp. Immunol. 9: 641-652.

Kuo, L.-M., and Robb, R.J. 1986a. Structure-function relationships for the IL-2 receptor system. I. Localization of a receptor binding site on IL-2. J. Immunol. 137: 1538-1543.

Kuo, L.-M., Rusk, C.M., and Robb, R.J. 1986b. Structure-function relationships for the IL-2 receptor system. II. Localization of an IL-2 binding site on high- and low-affinity receptors. J. Immunol. 137: 1544-1551.

Kurt-Jones, E.A., Kiely, J.M., and Unaue, E.R. 1985. Conditions required for expression of membrane IL-1 on B cells. J. Immunol. 135: 1548-1551.

Lamers, C.H.J., and de Haas, M.J.H. 1985. Antigen localization in the lymphoid organs of carp (*Cyprinus carpio*). Cell Tissue Res. 242: 491-498.

Lefkovits, I., and Waldman, H. 1979. Limiting dilution analysis of cells in the immune system. Cambridge: Cambridge University Press.

LePault, F., Coffman, R.L., and Weissman, I.L. 1983. Characteristics of thymus homing bone marrow cells. J. Immunol. 131: 64-69.

Levitt, D. and Cooper, M.D. 1980. Mouse pre-B cells synthesize and secrete m heavy chains but not light chains. Cell. 19: 617-625.

Lotze, M.T., Frana, L.W., Sharrow, S.O., Robb, R.J., and Rosenberg, S.A. 1985. *In vivo* administration of purified human IL-2. I. Half-life and immunologic effects of the Jurkat cell-line derived IL-2. J. Immunol. 134: 157-166.

Lowenthal, J.W., Cerottini, J.C., and MacDonald, H.R. 1986. Interleukin 1-dependent induction of both interleukin 2 secretion and interleukin 2 receptor expression by thymoma cells. J. Immunol. 137: 1226-1231.

Mancilla, J., Ikejima, T., and Dinarello, C.A. 1992. Glycosylation of the interleukin-1 receptor type I is required for optimal binding of interleukin-1. Lymph.-Cyt. Res. 11: 197-205.

March, C.J., Mosely, B., Larsen, A., Cerretti, D.P., Braedt, G., Price, V., Gilis, S., Henney, C.S., Kronheim, S., Grabstein, K.H., Colon, P., Hopp, T., and Cosman, D. 1985. Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. Nature 315: 641-647.

Marumo, K., Tachibana, M., Deguchi, N., and Tazaki, H. 1992. Enhancement of lymphokine-activated killer activity induction *in vitro* by interleukin-1 administered in patients with urological malignancies. J. Immunother. 11: 191-197.

Matsushima, K.A., Copeland, T.D., Onozaki, K., and Oppenheim, J.J. 1986. Purification and biochemical characteristics of two distinct forms of interleukins 1 from the myelomonocytic THP-1 cell line. Biochemistry 25: 3424-3429.

Matsushima, K.A., Procopio, A., Abe, H., Scala, G., Ortaldo, J.R., and Oppenheim, J.J. 1985. Production of interleukin-1 activity by normal human peripheral blood B lymphocytes. J. Immunol. 135: 1132-1136.

McKinney, E.C., Ortiz, G., Lee, J.C., Sigel, M.M., Lopez, D.M., Epstein, R.S., and McLeod, T.F. 1977. Lymphocytes of fish: Multipotential or specialized? In: Wright, R.K., and Cooper, E.L. (editors) Phylogeny of thymus and bone marrow-bursa cells. Elsevier/North-Holland Biomedical Press. Amsterdam, The Netherlands. Pages 73-82.

McMahan, C.J., Slack, J.L, Mosley, B., Cosman, D., Jenkins, J.M., Brannan, C.I., and Bomsztyk, K. 1991. A novel IL-1 receptor, cloned from B cells by mammalian expression, is expressed in many cell types. EMBO J. 10: 2821-2832.

Melief, C.J.M. 1989. Dendritic cells as specialized antigen-presenting cells. Res. Immunol. 140: 902-906.

Miller, N.W., Bly, J.E., van Ginkel, F.W., Ellsaesser, C.F., and Clem, L.W. 1987. Phylogeny of lymphocyte heterogeneity: Identification and separation of functionally distinct subpopulations of channel catfish lymphocytes. Dev. Comp. Immunol. 11: 739-747.

Miller, N.W., and Clem, L.W. 1984. Temperature-mediated processes in teleost immunity: Differential effects of temperature on catfish *in vitro* primary and secondary responses to thymus-dependent and thymus-independent antigens. J. Immunol. 133: 2356-2359.

Miller, N.W., Deuter, A., and Clem, L.W. 1986. Phylogeny of lymphocyte heterogeneity: The cellular requirements for the mixed leucocyte reaction with channel catfish. Immunology 59: 123-128.

Miller, N.W., Sizemore, R.C., and Clem, L.W. 1985. Phylogeny of lymphocyte heterogeneity: The cellular requirements for *in vitro* antibody responses of channel catfish leukocytes. J. Immunol. 134: 2884-2888.

Mizuochi, T., McKean, D.J., and Singer, A. 1988. IL-1 as a cofactor for lymphokine-secreting CD8+ murine T cells. J. Immunol. 141: 1571-1575.

Munakata, T., Semba, U., Kuwano, K., and Arai, S. 1985. Induction of interferon-gamma production by human natural killer cells stimulated by hydrogen peroxide. J. Immunol. 134: 2449-2455.

Nakanishi, K., Hashimoto, T., Hiroishi, K., Matsui, K., Yoshimoto, T., Morse, H.C.I., Furuyama, J.-I., Hamaoka, T., Higashino, K., and Paul, W.E. 1987. Demonstration of upregulated IL-2 receptor expression on an *in vitro* cloned BCL1 subline. J. Immunol. 138: 1817-1825.

Namen, A.E., Lupton, S., Hjerrild, K., Wignalol, J., Mochizuki, D.Y., Schmierer, A., Mosley, B., March, C.J., Urdal, D., Gillis, S., Cosman, D., and Goodwin, R.G. 1988. Stimulation of B cell progenitors by cloned murine interleukin-7. Nature 333: 571-573.

Neeper, M.P., Kuo, L.-M., Kieffer, M.C., and Robb, R.J. 1987. Structure-function relationships for the IL-2 receptor system. III. Tac protein missing amino acids 102 to 173 is unable to bind IL-2: Detection of spliced protein after L cell transfection. J. Immunol. 138: 3532-3538.

Neta, R., Oppenheim, J.J., and Durum, S.K. 1990. The cytokine concept: Historical perspectives and current status of the cloned cytokines. In: Cohen, S. (editor). Lymphokines and the immune response. CRC Press, Boca Raton, FL. Pages 29-42.

Neta, R., Oppenheim, J.J., Schreiber, R.D., Chizzonite, R., Ledney, G.D., and MacVitte, T.J. 1991. Role of cytokines (IL-1, TNF, and TGF beta) in natural and lipopolysaccharide-enhanced radioresistance. J. Exp. Med. 173: 1177-1182.

Nikaido, T., Shimizu, A., Ishida, N., Sabe, H., Teshigarawa, K., Maeda, M., Uchiyama, T., Todoi, J., and Honjo, T. 1984. Molecular cloning to cDNA encoding human interleukin 2 receptor. Nature 311: 631-635.

Nilsson, M., and Berg, T. 1977. Uptake and degradation of formaldehyde-treated ^{125}I-labelled human serum albumin in rat liver cells *in vivo* and *in vitro*. Biochem. Biophys. Acta 497: 171-182.

Nuchtern, J.G., Biddison, W.E., and Klausner, R.D. 1990. Class II MHC molecules can use the endogenous pathway of antigen presentation. Nature 343: 74-76.

Oka, M., and Ito, Y. 1987. Polyclonal B-cell-activating factors produced by spleen cells of mice stimulated with a cell homogenate of *Trypanosoma gambiense*. Infect. Immunol. 55: 3162-3167.

Okamoto, N., Shirakura, T., Nagakura, Y., and Sano, T. 1983. The mechanism of interference with fish viral infection in the FTG-2 cell line. Fish Pathol. 18: 7-12.

Oliver, K., Noelle, R.J., Uhr, J.W., Krammer, P.H., and Vitetta, E.S. 1985. B-cell growth factor (B cell growth factor I or B-cell-stimulating factor, provisional 1) is a differentiation factor for resting B cells and may not induce cell growth. Proc. Natl. Acad. Sci. 82: 2465-2467.

Oppenheim, J.J., Kovacs, E.J., Matsushima, K., and Durum, S.K. 1986. There is more than one interleukin-1. Immunol. Today 7: 45-48.

Ortega, H.W., and Kaattari, S.L. 1993a. Mechanisms of accessory function in trout (*Oncorhynchus mykiss*). I. Requisite signals for B cell antibody production to a T-independent antigen. Submitted for publication.

Ortega, H.W., and Kaattari, S.L. 1993b. Mechanisms of accessory function in trout (*Oncorhynchus mykiss*). II. Induction of cytokines and their receptors during antigen-specific and polyclonal responses. Submitted for publication.

Osmond, D.G. 1986. Population dynamics of bone marrow B lymphocytes. Immunol. Rev. 93: 103.

Owen, J.J. 1972. The origins and development of lymphocyte populations. In: Ontogeny of acquired immunity. Assoc. Scient. Publ. Amsterdam, The Netherlands. Pages 35-64.

Owen, J.J., Wright, D.E., Habu, S., Raff, M.C., and Cooper, M.D. 1977. Studies on the generation of B lymphocytes in fetal liver and bone marrow. J. Immunol. 118: 2067-2072.

Papadimitriou, J.M., and Ashman, R.B. 1989. Macrophages: Current views on their differentiation, structure and functions. Ultrastruct. Pathol. 13: 343-372.

Paul, N.L., and Ruddle, N.H. 1988. Lymphotoxin. Ann. Rev. Immunol. 6: 407-438.

Paul, W.E., and Ohara, J. 1987. B cell stimulatory factor-1/interleukin 4. Ann. Rev. Immunol. 5: 429-459.

Pestka, S., Langer, J.A., Zoon, K.C., and Samuel, C.E. 1987. Interferons and their actions. Ann. Rev. Biochem. 56: 727-777.

Pike, B.L., and Nossal, G.J.V. 1985. Interleukin-1 can act as a B cell growth and differentiation factor. Proc. Nat. Acad. Sci. 82: 8153-8159.

Pontius, H., and Ambrosius, H. 1972. Contribution to the immune biology of poikilothermic vertebrates. IX. Studies on the cellular mechanism of humoral immune reactions in perch *Perca fluviatilis* L. Acta Biol. Med. Ger. 29: 319-339.

Pure, E., Isakson, P., Kappler, J., Marrack, P., Krammer, P., and Vitteta, E. 1983. T cell-derived growth and differentiation factor. Dichotomy between the responsiveness of B cells from adult and neonatal mice. J. Exp. Med. 157: 600-608.

Rabin, E.M., O'Hara, J., and Paul, W.E. 1985. B-cell stimulatory factor 1 activates resting B cells. Proc. Natl. Acad. Sci. 82: 2935-2939.

Radeke, H.H., Martin, M., Topley, N., Kaever, V., and Resch, K. 1991. Differential biological activities of human interleukin-1 alpha and beta. Eur. Cytokine Netw. 2: 51-59.

Raff, M.C., Megson, M., Owen, J.J., and Cooper, M.D. 1976. Early production of intracellular IgM by B lymphocyte precursors in mouse. Nature. 259: 224-226.

Re, F., Mengozzi, M., Muzio, M., Dinarello, C.A., Mantovani, A., and Colotta, F. 1993. Expression of interleukin-1 receptor antagonist (IL-1ra) by human circulating polymorphonuclear cells. Eur. J. Immunol. 23: 570-573.

Reske-Kunz, A.B., Reske, K., and Rude, E. 1986. Cloned Ia+ BK-BI-2.6.C6 T cells function as accessory cells presenting protein antigen to long term cultured antigen specific T cell lines. J. Immunol. 36: 2033-2040.

Rhodes, J., Ivanyi, J., and Cozens, P. 1986. Antigen presentation by human monocytes: Effects of modifying class II antigen expression and IL-1 production by using recombinant interferons and corticosteroids. Eur. J. Immunol. 16: 370-375.

Rittenberg, M.B., and Amkraut, A.A. 1966. Immunogenicity of trinitrophenyl-hemocyanin: Production of primary and secondary anti-hapten precipitans. J. Immunol. 87: 421-430.

Rittenberg, M.B., and Pratt, K. 1969. Anti-Trinitrophenyl (TNP) plaque assay. Primary responses of BALB/c mice to soluble and particulate immunogen. Proc. Soc. Exp. Biol. Med. 132: 575-581.

Robb, R.J., and Greene, W.C. 1983. Direct demonstration of the identity of T cell growth factor binding protein and the Tac antigen. J. Exp. Med. 158: 1332-1337.

Robb, R.J., Greene, W.C., and Rusk, C.M. 1984. Low and high affinity cellular receptors for interleukin 2. Implications for the level of Tac antigen. J. Exp. Med. 160: 1126-1146.

Robb, R.J., Kutny, R.M., and Chowdry, V.C. 1983. Purification and partial sequence analysis of human T cell growth factor. Proc. Nat. Acad. Sci. 80: 5990-5994.

Robertson, O.H., and Wexler, B.C. 1960. Histological changes in the organs and tissues of migrating and spawning Pacific salmon (genus *Oncorhynchus*). Endocrinology 66: 222-239.

Rock, K.L., Gamble, S., and Rothstein, L. 1990. Presentation of exogenous antigen with class I major histocompatibility complex molecules. Science 249: 918-921.

Rombout, J.H.W.M., and van den Berg, A.A. 1989. Immunological importance of the second gut segment of carp I. Uptake and processing of antigens by epithelial cells and macrophages. J. Fish Biol. 35: 13-22.

Rombout, J.H.W.M., van den Berg, A.A., van den Berg, C.T.G.A., Witte, P., and Egberts, E. 1989. Immunological importance of the second gut segment of carp III. Systemic and/or mucosal immune responses after immunization with soluble or particulate antigen. J. Fish Biol. 35: 179-186.

Ruben, L.N., Warr, G.W., Decker, J.M., and Marchalonis, J.J. 1977. Phylogenetic origins of immune recognition: Lymphoid heterogeneity and the hapten/carrier effect in the goldfish *Carassius auratus*. Cell. Immunol. 31: 266-283.

Sabath, D.E., and Prystowsky, M.B. 1990. The molecular basis of interleukin 2 action. In: Cohen, S. (editor). Lymphokines and the immune response. CRC Press, Boca Raton, FL. Pages 181-198.

Scala, G., Kuang, Y.D., Hall, E., Muchmore, A.V., and Oppenheim, J.J. 1984. Accessory cell function of human B cells. I. Production of both interleukin-1 like activity and an interleukin-1 inhibitory factor by an EBV-transformed human B cell line. J. Exp. Med. 159: 1637-1642.

Schrader, J.W. 1986. The panspecific hemopoietin of activated lymphocytes (interleukin 3). Ann. Rev. Immunol. 4: 205-230.

Schreiber, R.D., Pace, J.L., Altman, A., and Katz, D.H. 1983. Macrophage-activating factor produced by a T cell hybridoma: Physiochemical and biosynthetic resemblance to gamma-interferon. J. Immunol. 131: 826-832.

Secombes, C.J. 1990. Isolation of salmonid macrophages and analysis of their killing activity. In: Stolen, J.S., Fletcher, T.C., Anderson, D.P., Robertson, B.S., and van Muiswinkel, W.B. (editors) Techniques in Fish Immunology. SOS Publications, Fair haven, NJ. Pages 137-154.

Secombes, C.J. 1987. Lymphokine release from rainbow trout leukocytes stimulated with Concanavalin A. Effects upon macrophage spreading and adherence. Dev. Comp. Immunol. 11: 513-520.

Secombes, C.J., and Fletcher, T.C. 1992. The role of phagocytes in the protective mechanisms of fish. Ann. Rev. Fish Dis. 2: 53-72.

Secombes, C.J., Manning, M.J., and Ellis, A.E. 1982. Localization of immune complexes and heat-aggregated immunoglobulin in the carp *Cyprinus carpio L.* Immunology 47: 101-105.

Semple, J.W., Ellis, J., and Delovitch, T.L. 1989. Processing and presentation of insulin II. Evidence for intracellular, plasma-membrane associated and extracellular degradation of human insulin by antigen presenting B cells. J. Immunol. 142: 4184-4193.

Sigel, M.M., Hamby, B.A., and Huggins, E.M. 1986. Phylogenetic studies on lymphokines. Fish lymphocytes respond to human IL-1 and epithelial cells produce an IL-1-like factor. Vet. Immunol. Immunopath. 12: 47-58.

Sima, P., and Vetvicka, V. 1990. Evolution of Immune Reactions. CRC Press. Boca Raton, Florida. Pages 171-227.

Simon, P.L. and Lee, J.C. 1986. Evidence for shared antigenic determinants on rabbit and human interleukin-1 (IL-1). J. Immunol. 137: 557-562.

Sinha, A.A., Guidos, C., Lee, K.C.,and Diener, E. 1987. Functions of accessory cells in B cell responses to thymus-independent antigens. J. Immunol. 138: 4143-4149.

Sizemore, R.C., Miller, N.W., Cuchens, M.A., Lobb, C.J., and Clem, L.W. 1984. Phylogeny of lymphocyte heterogeneity: The cellular requirements for *in vitro* mitogenic responses of channel catfish leukocytes. J. Immunol. 133: 2920-2924.

Smedsrud, T., Dannevig, B.H., Tolleshaug, H., and Berg, T. 1984. Endocytosis of a mannose-terminated glycoprotein and formaldehyde-treated human serum albumin in liver and kidney cells from fish (*Salmo alpinus*). Dev. Comp. Immunol. 8: 579-588.

Smith, A.M., Potter, M. and Merchant, E.B. 1967. Antibody-forming cells in the pronephros of the teleost *Lepomis macrochirus.* J. Immunol. 99: 876-882.

Smith, A.M., Wivel, N.A., and Potter, M. 1970. Plasmacytosis in the pronephros of the carp Cyprinus carpio. Anat. Rec. 167: 351-370.

Smith, K.A. 1988. Interleukin 2: Inception, impact, and implications. Science 240: 1169-1176.

Smith, K.A. 1984. Interleukin 2. Ann Rev. Immunol. 2: 319-333.

Smith, P.D., and Braun-Nesje, R. 1982. Cell mediated immunity in the salmon: Lymphocyte and macrophage stimulation, lymphocyte/macrophage interactions, and the production of lymphokine-like factors by stimulated lymphocytes. Dev. Comp. Immunol. Suppl. 2: 233-238.

Spriggs, M.K., Nevens, P.J., Grabstein, K., Dower, S.K., Cosman, D., Armitage, R.J., McMahan, C.J., and Sims, J.E. 1992. Molecular characterization of the interleukin-1 receptor on monocytes and polymorphonuclear cells. Cytokine 4: 90-95.

Stewart, W.E. 1980. Interferons: Several questions and few answers. Adv. Exp. Med. 166: 15-21.

Streilein, J.W., and Grammer, S.F. 1989. *In vitro* evidence that Langerhans cells can adopt two functionally distinct forms capable of antigen presentation to T lymphocytes. J. Immunol. 143: 3925-3933.

Strieter, R.M., Kasahara, K., Allen, R.M., Standiford, T.J., Rolfe, M.W., Becker, F.S., Chensue, S.W., and Kunkel, S.L. 1992. Cytokine-induced neutrophil-derived interleukin-8. Am. J. Pathol. 141: 397-407.

Szakal, A.K., Holmes, K.L., and Tew, J.G. 1983. Transport of immune complexes from the subcapsular sinus to lymph node follicles on the surface of nonphagocytic cells, including cells with dendritic morphology. J. Immunol. 131: 1714-1727.

Szakal, A.K., Kosco, M.H., and Tew, J.G. 1988. A novel *in vivo* follicular dendritic cell-dependent iccosome-mediated mechanism for delivery of antigen to antigen processing cells. J. Immunol. 140: 341-353.

Taniguchi, T. 1988. Regulation of cytokine gene expression. Ann. Rev. Immunol. 6: 439-464.

Taniguchi, T., Hatakeyama, M., Minamoto, S., Kono, T., Doi, T., Tsudo, M., and Miyasaka, M. 1990. Molecular analysis of the interleukin-2 receptor complex: Expression of the human alpha and beta chain cDNAs. Prog. Immunol. 7: 627-639.

Taniguchi, T., Mantei, N., Schwarzstein, M., Nagata, S., Muramatsu, M., and Weissman, C. 1980. Human IFN alpha and IFN beta are structurally related. Nature 285: 547-549.

Taniguchi, T., Matsui, H., Fujita, T., Takaoka, C., Kashima, N., Yoshimoto, R., and Hamuro, J. 1983. Structure and expression of a cloned cDNA for human interleukin-2. Nature 302: 305-311.

Tatner, M.F., and Manning, M.J. 1983. Growth of the lymphoid organs in rainbow trout *Salmo gairdneri* from one to fifteen months of age. J. Zool. 199: 503-520.

Tew, J.G., Kosco, M.H., Burton, G.F., and Szakal, A.K. 1988. Induction of the secondary antibody respons: Immune complex formation, iccosome release by folicular dendritic cells, processing and presentation of antigen by germinal center B cells and tingible body macrophages. In: Schook, L.B., Tew, J.G. (editors) Antigen presenting cells: Diversity, differentiation, and regulation. Alan R. Liss, New York. Pages 1-10.

Teyton, L., O'Sullivan, D., Dickson, P.W., Lotteau, P., Sette, A., Fink, P., and Peterson, P.A. 1990. Invariant chain distinguishes between exogenous and endogenous antigen presentation pathways. Nature 348: 39-44.

Tigges, M.A., Casey, L.S., and Koshland, M.E. 1989. Mechanism of interleukin-2 signaling: Mediation of different outcomes by a single receptor and transduction pathway. Science 243: 781-786.

Tocci, M.J., Shapiro, J.A., Cameron, P., Limjucco, G., Rodkey, J., Bennett, C., and Schmidt, J.A. 1985. Molecular biology of normal interleukin-1. Nature 314: 266-271.

Trinchieri, G., and Perussia, B. 1985. Immune interferon: A pleiotropic lymphokine with multiple effects. Immunol. Today 6: 131-136.

Tripp, R.T. 1988. Glucocorticoid regulation of salmonid B lymphocytes. Doctoral Thesis, Oregon State University. Corvallis, Oregon.

Unanue, E.R. 1984. Antigen-presenting cell function of the macrophage. Ann. Rev. Immunol. 2: 395-428.

Vallejo, A.N., Ellsaesser, C.F., Miller, N.W., and Clem. L.W. 1991. Spontaneous development of functionally active long term monocyte-like cells lines from channel catfish. *In Vitro* Cell. Dev. Bio. 27A: 279-286.

Vallejo, A.N., Miller, N.W., and Clem, L.W. 1992. Cellular pathway(s) on antigen processing in fish APC: Effects on varying *in vitro* temperatures on antigen catabolism. Dev. Comp. Immunol. 16: 25-31.

Vallejo, A.N., Miller, N.W., Jorgensen, T., and Clem, L.W. 1990. Phylogeny of immune recognition: Antigen processing/presentation in channel catfish immune responses to hemocyanins. Cell. Immunol. 130: 362-377.

van Rooijen, N. 1990. Antigen processing and presentation *in vivo*: The microenvironment is a crucial factor. Immunol. Today 11: 436-439.

Venkataraman, M., and Scott, D.W. 1977. Cellular events in tolerance. VI. Neonatal vs adult B cell tolerance: Differences in antigen-binding cell patterns and lipopolysaccharide stimulation. J. Immunol. 119: 1879-1884.

Weaver, C.T., and Unanue, E.R. 1990. The costimulatory function of antigen presenting cells. Immunol. Today 11: 49-54.

Weitzman, M.N., and Savage, N. 1992. Nuclear internalization and DNA binding activities of interleukin-1 receptor and interleukin-1/receptor complexes. Biochem. Biophys. Res. Commun. 187: 1166-1171.

Welte, K., Wang, C.Y., Mertelsmann, R., Venuta, S., Feldman, S.P., and More, M.A.S. 1982. Purification of human interleukin 2 to apparent homogeneity and its molecular heterogeneity. J. Exp. Med. 156: 454-460.

Wheelock, E.F. 1965. Interferon-like virus-inhibitor induced in human leukocytes by phytohemagglutinin. Science 149: 310-311.

White, R.G., Henderson, D.C., Eslami, M.B., and Nielsen, K.H. 1975. Localization of a protein antigen in the chicken spleen. Effect of various manipulative procedures on the morphogenesis of the germinal center. Immunology 28: 1-21.

Whitlock, C., Denis, K., Robertson, D., and Witte, O. 1985. *In vitro* analysis of murine B-cell development. Ann. Rev. Immunol. 3: 213-235.

Wilson, B., Jeffreys, A.J., Barrie, P.A., Boseley, P.G., Slocombe, P.M., Easton, A., and Burke, D.C. 1983. A comparison of vertebrate interferon gene families detected by hybridization with human interferon DNA. J. Mol. Biol. 166: 457-475.

Yamaga, K.M., Kubo, R.T., and Etlinger, H.M. 1978. Studies on the question of conventional immunoglobulin on thymocytes from primitive vertebrates. II. Delineation between Ig-specific and cross-reactive membrane components. J. Immunol. 120: 2074-2079.

Yasutake, W.T., and Wales, J.H. 1983. Microscopic anatomy of salmonids: An atlas. Resource Publication 150: 82-99.

Yewdell, J.W., and Bennick, J.R. 1990. The binary logic of antigen processing and presentation to T cells. Cell 62: 203-206.

Zapata, A. 1981. Lymphoid organs of teleost fish. I. Ultrastructure of the thymus of *Rutilus rutilus*. Dev. Comp. Immunol. 5: 427-436.

Zapata, A. 1979. Ultrastructural study of the teleost fish kidney. Dev. Comp. Immunol. 3: 55-65.

Zapata, A. G., and Cooper, E.L. 1990. The bone marrow and its equivalents. In: The immune system: Comparative histophysiology. Pages 35-71.

Zapata, A.G., Torroba, M., Alvarez, F., Anderson, D.P., Dixon, O.W., and Wisniewski, M. 1987. Electron microscopic examination of antigen uptake by salomind gill cells after bath immunization with a bacterin. J. Fish Biol. 31A: 209-217.

Zarcone, D., Prasthofer, E.F., Malavasi, F., Pistoia, V., LoBuglio, A.F., and Grossi, C.E. 1987. Ultrastructural analysis of human natural killer cell activation. Blood 69: 1725-1736.

Ziegenfuss, M.C., and Wolke, R.E. 1991. The use of fluorescent microspheres in the study of piscine macrophage aggregate kinetics. Dev. Comp. Immunol. 15: 165-171.

Zoja, C., Bettoni, S., Morigi, M., Remuzzi, G., and Rambaldi, A. 1992. Interleukin-1 regulates cytokine gene expression in human mesanglial cells through the interleukin-1 receptor type 1. J. Am. Soc. Nephrol. 2: 1709-1715.

Zola, H., Flego, L., Wong, Y.T., Macardle, P.J., and Kenney, J.S. 1993. Direct demonstration of membrane IL-1 alpha on the surface of circulating B lymphocytes and monocytes. J. Immunol. 150: 1755-1762.

APPENDICES

APPENDIX 1

Reagents and Buffers

Benzocaine. Fish were anesthetized by immersion in a benzocaine bath of approximately 2 ml stock solution/4 liters of water. The stock solution was prepared by dissolving 10 grams ethyl-p-aminobenzoate in 100 mls of 95% ethanol (Kaattari and Irwin, 1985).

Cacodylate buffer. A solution of 0.28 M Cacodylate buffer was prepared as described by Rittenberg and Amkraut (1966), by dissolving 3.82 grams of cacodylic acid (Sigma, St. Louis, MO) in 100 ml distilled water. 1N sodium hydroxide was used to adjust the pH to 7.0. Cacodylate buffer was stored at 4°C for no longer than one month.

Modified Barbitol Buffer (MBB). A stock solution of 5X MBB was prepared by dissolving 1 vial (0.05 moles sodium barbital, 0.01 moles barbital) barbital buffer (Sigma) in one liter of distilled water at room temperature with constant stirring. Anhydrous calcium chloride (0.083g/l), magnesium chloride (0.508 g/l), and sodium chloride (42.5 g/l) were then added, and the pH adjusted to 7.4. The 5X stock solution was autoclaved and stored at 17°C. A 1X working solution was prepared by dilution with filter sterilized saline (8.7 g/l).

Phosphate buffered saline. Phosphate buffered saline (PBS) was prepared by dissolving 1 gram of monobasic potassium phosphate (KH_2PO_4) and 17.8 grams of dibasic sodium phosphate-7 hydrate

(Na_2HPO_4-$7H_2O$) in one liter of distilled water. To this solution 8.5 grams of sodium chloride was added and the pH adjusted to 7.4.

Trinitrophenylated lipopolysaccharide (TNP-LPS). TNP-LPS was prepared by the method of Jacobs and Morrison (1975). 161 mg of *E. coli* serotype 055.B5 lipopolysaccharide (Sigma) was suspended in cacodylate buffer (8.05 ml), with constant stirring. The pH of the solution was adjusted to 11.5 with 10 N sodium hydroxide. The tube was foil-wrapped and picrylsulfonic acid (96mg dissolved in 8ml cacodylate buffer) was added dropwise with constant stirring. The solution was stirred for two hours at room temperature. This solution of TNP-LPS was then dialyzed against 4 (1 liter) changes of saline, and one (1 liter) change of RPMI-1640. TNP-LPS was pasteurized for 45 minutes at 70°C. Stock TNP-LPS was stored at 4°C, and diluted in RPMI just prior to use.

Complement. Sera was obtained from spawned adult steelhead trout and used as the source of complement. Blood was collected in 50 ml centrifuge tubes, held on ice and allowed to clot overnight at 4°C. The sera was removed and pooled, then divided into aliquots which were stored at -70°C until used. Complement was diluted with MBB prior to use in the plaque forming cell assay.

APPENDIX 2

Sheep Red Blood Cell (SRBC) Haptenation

Trinitrophenylated SRBCs were prepared using a modification of a method described by Rittenberg and Pratt (1969). SRBCs were washed 3X in 1X MBB by centrifugation for 5 minutes at 500 x g. To the wet packed SRBCs (1 ml), a solution of 200 μl picrylsulfonic acid in 3.3 ml cacodylate buffer was added dropwise. The tube was foil-wrapped and placed on a rotator for 20 minutes at room temperature. The SRBC solution was centrifuged, the supernatant removed and a solution of 0.0037 g of glycyl-glycine dissolved in 5.8 ml MBB was added to block the remaining active sites. The cells were centrifuged again, supernatant removed, then washed 3X in MBB. The SRBCs were then resuspended to a concentration of 20% (by volume) in MBB for final dilution.